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Amplification of the HER-2 gene often leads to breast cancer by causing cells to make abnormally high levels of the wild-type HER-2 protein. Evidence now shows that the interaction between HER-2 and HER-3 leads to the constitutive activation of HER-2/HER-3 heterodimers in breast cancer cells with HER-2 gene amplification, and HER-2/HER-3 potently activates multiple signal transduction pathways involved in mitogenesis. This indicates that inhibition of the interaction between HER-2 and HER-3 may be an especially effective and unique strategy for blocking the effects of HER-2 in human breast cancer cells. Therefore, we constructed a bicistronic retroviral expression vector that codes for a dominant negative form of HER-3 that can inactivate the function of HER-2/HER-3. Dominant negative HER-3 also specifically inhibited proliferation induced by heregulin (the ligand for HER-2/HER-3) as well as the growth factor-independent (i.e. autonomous) proliferation and anchorage-independent growth of breast cancer cells with HER-2 gene amplification. We now propose to use dominant negative HER-3 vectors in experiments to determine the effectiveness of dominant negative HER-3 for blocking HER-2/HER-3 activation, signaling and growth in culture and *in vivo* for different breast cancer cells lines.

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INTRODUCTION

The HER-2 (*neu*/*erb*B-2) gene encodes a 185 kDa protein tyrosine kinase that is highly homologous to the epidermal growth factor (EGF) receptor (EGFR/HER-1/*erb*B- 1), HER-3 (*erb*B-3), and HER-4 (*erb*B-4) (1-3), which together, comprise the type 1 family of receptor tyrosine kinases (4, 5). These receptors differ in their ligand specificities (4), and while HER-1 binds several ligands closely related to EGF, HER-3 and HER-4 are the receptors for a number of different isoforms of *neu* differentiation factor/heregulin (HRG) (6-8). While no direct ligand for HER-2 has yet been cloned, it is now clear that HER-2 is capable of heterodimerization with HER-1 (9, 10), HER-3 (11, 12), or HER-4 (8), and these HER-2-containing heterodimers form the highest affinity binding sites for their respective ligands (10, 11). HER-2 is amplified in 28% of primary breast carcinomas *in vivo* (13), and another 10% overexpress HER-2 without amplification of the gene (14-16). HER-2 gene amplification, concordant with high-level overexpression, is associated with increased tumor aggressiveness and the poor prognosis of breast cancer patients (13, 14, 17-19). Experimentally elevated HER-2 gene expression in various cell lines induces tumorigenesis in nude mice (24-27), and the potent oncogenic potential of HER-2 is generally thought to be due to its ability to constitutively activate key signaling pathways that are involved in the regulation of cell growth (28). Although HER-2 was originally discovered as the *neu* transmembrane-mutated form of the gene in rat neuroblastoma cells (29), the HER-2 gene found in human breast cancer cells has never shown such mutations (30). The level of tyrosine-phosphorylated HER-2 in primary breast cancer *in vivo* always shows a direct correspondence with the HER-2 overexpression (31), and while the wild-type HER-2 protein possess constitutive tyrosine kinase activity when overexpressed in cell lines in the absence of any identifiable ligand (24-27, 32, 33), the HER-2 tyrosine kinase domain is also constitutively active in EGFR-HER-2 chimeric receptors in the absence of EGF (32, 33). Therefore, the overexpression of wild-type HER-2 alone is sufficient to constitutively activate its tyrosine kinase function. Additionally, heterodimeric interactions are now known to occur between the different HER proteins, including HER-1 and HER-2 (9, 10), HER-2 and HER-3 (11, 12), HER-2 and HER-4 (8), and HER-1 and HER-3 (34, 35). Our own work and that of others has now shown that heterodimer interactions between HER-2 and HER-3 are constitutively active in breast cancer cells with HER-2 gene amplification (20-23), and co-transfection of HER-3 with HER-2 greatly augments the transforming capability of HER-2 in genetically engineered cell lines (21). Therefore, we are particularly interested in how the cooperative effects of HER-2/HER-3 heterodimers activate key mitogenic signaling pathways which facilitate cell growth. Our own work and that of others has also now shown that the constitutive activation of HER-2/HER-3 in breast cancer cells is associated with the constitutive activation of phosphatidylinositol (PI) 3-kinase and mitogen-activated protein (MAP)-kinase (20-22). One strategy that has been used successfully to block the function of other receptor tyrosine kinases employs dominant negative expression vectors in which a region coding for the cytoplasmic domain of the receptor is almost completely removed. While the truncated receptor still contains the extracellular and transmembrane domains and can dimerize within the cell membrane, it lacks tyrosine kinase activity and inhibits the signal transduction docking function of both mutant/mutant homodimers and mutant/wild-type heterodimers (Fig. 1A). This strategy has been used effectively to block EGFR (36), platelet-derived growth factor receptor (37), and fibroblast growth factor receptor (38) in biochemistry studies. Recently, a dominant negative HER-2 vector was also used successfully to block HER-2

function in normal mouse development (40). The use of such dominant negative HER-2 vectors has not yet been reported to block HER-2 in cancer cells with HER-2 gene amplification, and this is likely due to the stoichiometric problems associated with the inability to generate mutant/wild-type levels high enough for effective inhibition in human breast cancer cells with HER-2 gene amplification. However, the fact that HER-3 is not highly overexpressed in these cells, and that activated HER-2/HER-3 has a particularly high-affinity interaction (42-44), suggests that dominant negative HER-3 may be especially effective in blocking HER-2/HER-3 function. Therefore, we have introduced a dominant negative form of HER-3 (Fig. 1B) into cells in an attempt to block the activation of HER-2/HER-3 (45).

BODY

We sought to determine the effectiveness of dominant negative HER-3 in blocking HER-2/HER-3 activation, signaling and the transformed growth of breast cancer cells with HER-2 gene amplification. We routinely use the H16N-2 normal breast epithelial cells and the 21MT-1 breast carcinoma cells for our studies because they were derived from the same patient and can be grown under precisely defined serum-free conditions in culture. This well defined system allows us to study growth factor-independent (i.e. autonomous) proliferation and responses to exogenous growth factors in a manner that is not yet possible for other cell lines derived in medium containing high levels of serum. Therefore, this serum-free system is ideal for studying receptor activation and cell signaling by allowing us to distinguish constitutive from externally mediated growth factor responses in culture (22, 41, 45). We had previously shown that the amplification and high-level overexpression of HER-2 in the 21MT-1 cells was associated with constitutive activation of HER-2/HER-3 and PI 3-kinase, as well as their growth factor independent proliferation in culture (22). H16N-2 and 21MT-1 cells were then infected with the control pCMV vector or pCMV-dn3 (Fig. 1B), selected on G418, and then used to determine the effects of dominant negative HER-3 on HER-2/HER-3 activation, signaling and growth responses in culture (45-see Appendices). Dominant negative HER-3 inhibited the constitutive activation of HER-2/HER-3 in 21MT-1-dn3 cells and the HRG-stimulated activation of HER-2/HER-3 in H16N-2-dn3 and 21MT-1-dn3 cells (45). Separate immunoprecipitations followed by immunoblotting showed that dominant negative HER-3 moderately inhibited the constitutive or HRG-stimulated tyrosine phosphorylation of HER-2, and potently inhibited HER-3 (45). Furthermore, the constitutive or HRG-stimulated activation of PI 3-kinase and SHC protein recruitment and phosphorylation were also inhibited by dominant negative HER-3 (45). Monolayer growth assays under serum-free conditions also showed that dominant negative HER-3 inhibited the growth factor-independent proliferation of the 21MT-1-dn3 cells in the complete absence of exogenous growth factors in culture (45), as well as the HRG-induced proliferation of both H16N-2-dn3 and 21MT-1-dn3 cells in culture (45). Dominant negative HER-3 also blocked the anchorage-independent growth of 21MT-1-dn3 cells in soft agarose culture (45).

Interestingly, dominant negative HER-3 had no apparent effect on the insulin/EGF-induced proliferation of either H16N-2-dn3 or 21MT-1-dn3 cells after they had been infected with pCMV-dn3 and selected on G418 (Fig. 2, 45). This result has important significance, because it indicates that dominant negative HER-3 preferentially inhibits HER-2/HER-3-mediated growth responses compared to those mediated by exogenous EGF. Therefore, the effects of pCMV-dn3 show selectivity by preferentially inhibiting growth responses involving HER-2/HER-3, such as those that are constitutively activated by HER-2 or induced by HRG. This suggests that HER-1/HER-3 may not be as effectively inhibited by dominant negative HER-3 as is HER-2/HER-3. While EGFR and HER-3 do interact in these and other cell lines (46-see Appendices), clearly the EGFR/HER-3 heterodimer interaction is weak compared to the HER-2/HER-3 heterodimer interaction (42-44). In order to further investigate these preferential effects of dominant negative HER-3 on HER-2/HER-3-mediated growth responses in the 21MT-1-derived cells, we have also compared the levels of tyrosine phosphorylation in cells induced by either HRG or EGF (Fig 3). In order to measure the level of receptor activation, we deprive the cells of growth factors for 48 hours in serum-free, growth factor-free medium and then directly extract the cells (i.e. the "constitutive" condition) or stimulate the cells with growth factor for 10 min at 37° C and then

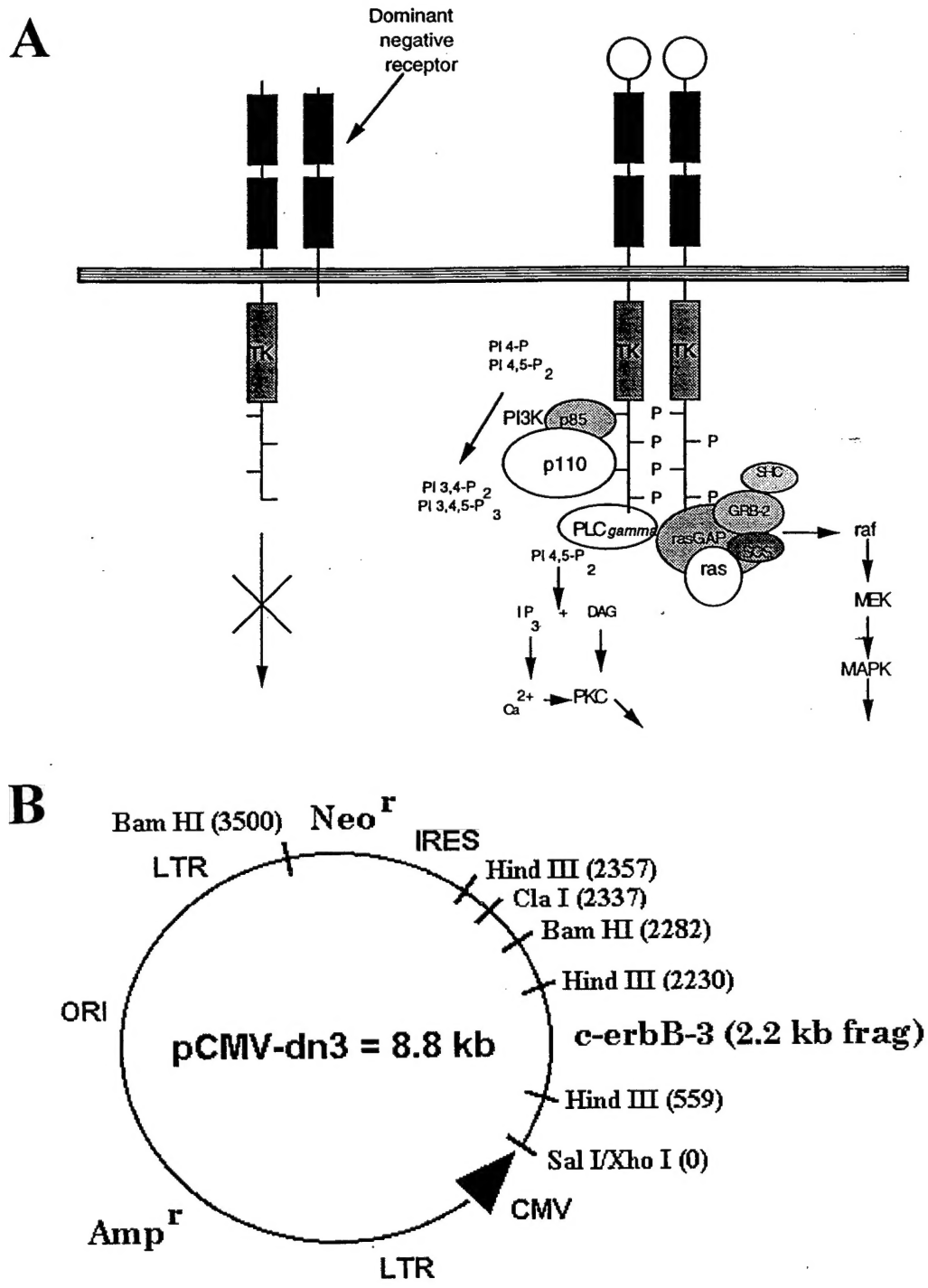

Fig. 1. *Bicistronic Retroviral Expression Vector for Dominant Negative HER-3.* (A) Diagram showing the strategy for blocking wild-type receptor tyrosine kinases with dominant negative (i.e. truncated) receptors. (B) The pCMV-dn3 bicistronic retroviral expression vector was constructed containing a dominant negative form of the HER-3 gene in which most of the cytoplasmic domain of HER-3 was removed. In addition, this vector contains an internal ribosome-binding site (IRES) between the HER-3 fragment and the neo^r gene located downstream, which together, form a single transcription unit when expressed in mammalian cells. The expression of ectopic genes in a bicistronic transcript then allows for the coordinate co-expression of the gene with antibiotic resistance in infected cells selected on antibiotic (45).

extract the cells for immunoprecipitation and/or Western blotting. While dominant negative HER-3 inhibited the constitutive tyrosine phosphorylation of HER-2/HER-3 as well as that seen with HRG stimulation, the EGF-induced tyrosine phosphorylation of EGFR-containing dimers was apparently not effected by dominant negative HER-3 under these conditions (Fig. 3). This result is consistent with the hypothesis that EGFR-containing dimers are not as effectively inhibited by dominant negative HER-3 as are HER-2/HER-3 heterodimers at a given level of dominant negative HER-3 in genetically engineered cells.

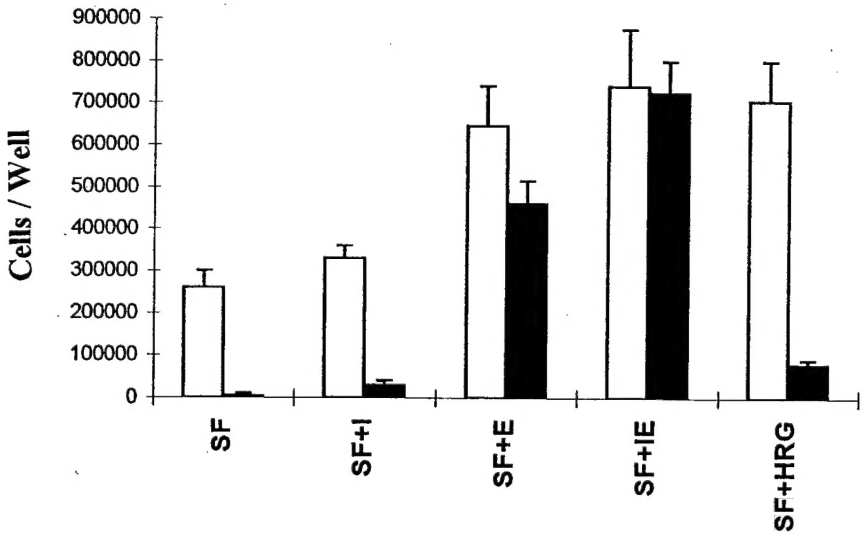

Fig. 2. *Dominant negative HER-3 inhibits the autonomous proliferation of 21MT-1 cells and HRG-induced proliferation in culture*. Anchorage-dependent monolayer growth assay showing the proliferation of 21MT-1 cells infected with pCMV (□) and 21MT-1 cells infected with pCMV-dn3 (■) in monolayer culture for 9 days with serum-free (SF) medium, plus insulin (I) and/or EGF (E), or plus HRG-β (HRG). The mean average and standard deviation for triplicate wells is shown.

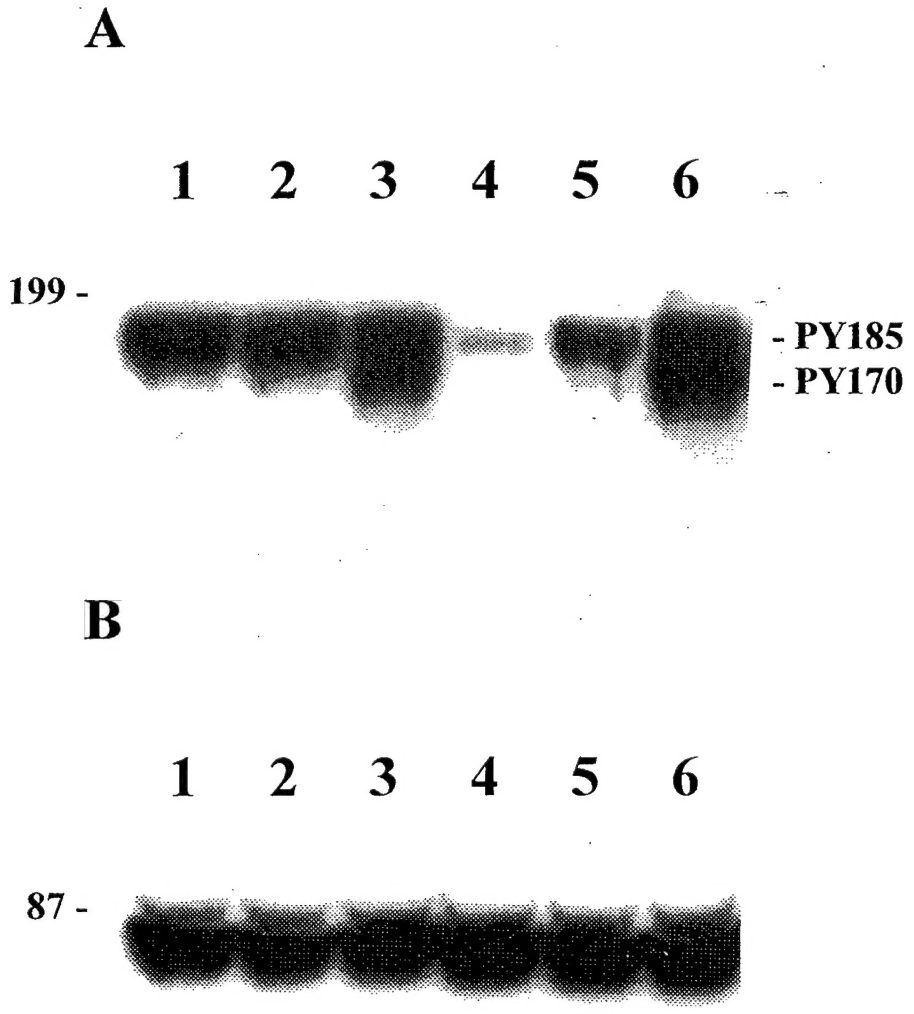

Fig. 3. *Preferential inhibition of HER-2/HER-3 tyrosine phosphorylation in 21MT-1 cells expressing dominant negative HER-3.* A, Samples containing 200 μg cell lysate protein per lane were immunoblotted with anti-phosphotyrosine antibody. B, The same blot was then reprobed with anti-p85 antibody as a control to confirm equal loading of the gel. 21MT-1 cells infected with pCMV (Lanes 1-3) or 21MT-1 cells infected with pCMV-dn3 (Lanes 4-6) were deprived of growth factors for 48 hours in serum-free, growth factor-free medium and then directly extracted (Lanes 1 and 4) or stimulated with 10 ng/ml HRG-β (Lanes 2 and 5) or 10 ng/ml EGF (Lanes 3 and 6) for 10 minutes at 37° C before cell lysate extraction.

In vivo studies were performed using the 21MT-1-derived cells for injections into nude mice as described in the grant proposal. However, the 21MT-1 cell line has not been sufficiently tumorigenic in our studies to allow us to sufficiently test the effects of dominant negative HER-3 *in vivo*. The limited growth seen for 21MT-1 cells in nude mice is apparently a common problem for a significant number of highly malignant and metastatic breast carcinoma cell lines (47), and earlier studies with 21MT-1 cells also suggested some difficulty in using these cells for tumor studies at later passage (48). We have not yet been able to detected tumors in nude mice or in Scid mice injected with 5×10^6 21MT-1 cells per injection. Experiments are also still in progress comparing injections done in both Nu/Nu and Nu/Nu CD-1 strains of nude mice. Studies have also now been initiated using BT-474, MDA-MB-453 and SK-BR-3 cells for injection into various immunodeficient mouse strains. These breast carcinoma cell lines have amplification of the HER-2 gene, and BT-474 and SK-BR-3 cells have been reported to form tumors in nude mice. Experiments are underway to compare the tumorigenesis of these cell lines in both Nu/Nu and Nu/Nu CD-1 strains of nude mice, as well as in Scid mice.

As outlined in the grant proposal, we have also infected BT-474, MDA-MB-453 and SK-BR-3 breast cancer cell lines with pCMV and pCMV-dn3 in order to study the effects of dominant negative HER-3 in a panel of different cell lines that have amplified HER-2 and have been reported to have constitutive activation of HER-3 (20). These cell lines are routinely cultured under less well defined serum-containing conditions than the 21MT-1 cells, but apparently have the advantage of being tumorigenic in nude mice. Titrations were initially performed to determine the concentrations of G418 necessary to kill BT-474, MDA-MB-453 and SK-BR-3 cells within 2-3 weeks after addition of the antibiotic, and these concentrations (i.e. 500 ug/ml for SK-BR-3 cells, 600 ug/ml for MDA-MB-453 cells and 700 ug/ml for BT-474 cells) were then used to select these cell lines on G418 after being infected with pCMV or pCMV-dn3. Infections of all three cell lines were first carried out using ψCRIP-derived packaging cell lines that were used previously to infect the H16N-2 and 21MT-1 cell lines (45). However, no colonies grew out for BT-474, MDA-MB-453 and SK-BR-3 cells infected with conditioned medium from ψCRIP-derived packaging cell lines. After no colonies were seen in pilot experiments, the BT-474, MDA-MB-453 and SK-BR-3 cells were then cultured in DMEM with 10% fetal bovine serum plus insulin and EGF, which we found maximally stimulates the growth of these cells in monolayer culture. In addition, the ψCRIP packaging cells are not thought to produce very high viral titers compared to other packaging cell lines. Therefore, we transfected the PA-317 packaging cell line with pCMV or pCMV-dn3 and then selected the transfected PA-317 cells on 800 ug/ml G418 for a month to derive packaging cell lines which were then used for infecting BT-474, MDA-MB-453 and SK-BR-3 cells. By using the PA-317-derived packaging cell lines, we have now successfully generated viral titers sufficient for good colony formation of BT-474, MDA-MB-453 and SK-BR-3 cells infected with pCMV (Fig. 4). Interestingly, we consistently saw a much lower number of colonies growing out for the cells infected with pCMV-dn3 under identical infection and culture conditions (Fig. 4). Furthermore, we noticed that many of the colonies that start to grow out in plates infected with pCMV-dn3 did not continue to grow. This suggests that the continued growth of many of the cells infected with pCMV-dn3 may be inhibited by the expression of dominant negative HER-3 during selection on G418.

In order to confirm the expression of dominant negative HER-3 in cells infected with pCMV-dn3, we performed anti-HER-3 immunocytochemistry on cells infected with pCMV or pCMV-dn3 after being selected on G418 (Fig. 5). The anti-HER-3 H105 antibody (Neomarkers)

is known to be highly specific for HER-3 and binds to an epitope within the extracellular domain of HER-3. While this antibody does not work for Western blotting, it works well for immunocytochemistry (45). While all of these cell lines express wild-type HER-3, the levels were relatively low when detected with HRP/DAB staining in cells infected with pCMV (Fig. 5A) compared to cells infected with pCMV-dn3 (Fig. 5B). Therefore, the high levels of HER-3 measured in cells infected with pCMV-dn3 confirmed the ectopic expression of dominant negative HER-3 in these cells. However, we have also noticed significant heterogeneity in the level of HER-3 staining in cell populations infected with pCMV-dn3 (Fig. 5B). We have previously shown that 100% of cell clones from cells infected with the pCMV bicistronic retroviral expression vector co-express the Lac Z marker gene with antibiotic resistance (45). However, heterogeneity in the level of expression of inserted genes is still seen between colonies. Therefore, while the different cell lines infected with pCMV-dn3 showed much higher levels of staining for HER-3 compared to controls, such heterogeneity in dominant negative HER-3 expression between different clones indicates that there is the potential for the preferential selection of those colonies expressing lower levels of dominant negative HER-3 if it inhibits the growth of these cells during selection on G418. As discussed in detail below, the difficulty of using a constitutive promoter for driving the expression of a gene which can inhibit growth may necessitate the use of an inducible system in order to directly determine the full effects of dominant negative HER-3 in cells with HER-2 gene amplification.

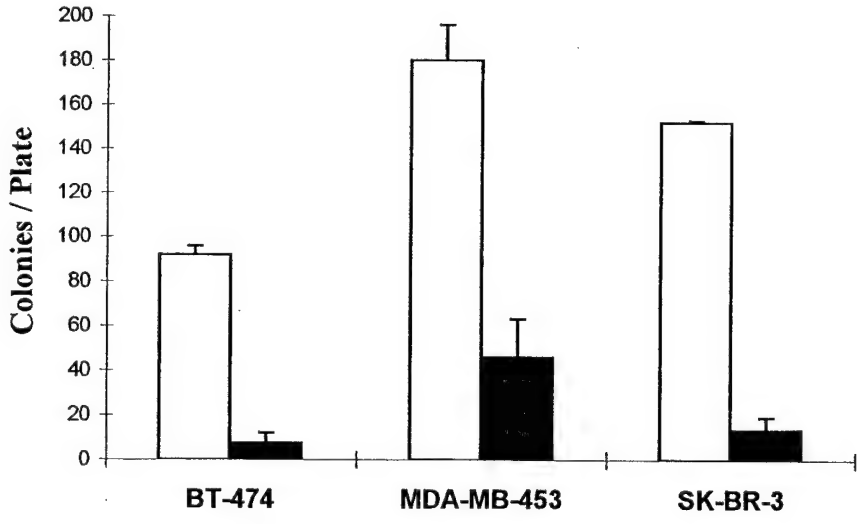

Fig. 4. *Clonal outgrowth of BT-474, MDA-MB-453 and SK-BR-3 cells infected with pCMV or pCMV-dn3.* BT-474, MDA-MB-453 and SK-BR-3 cells were infected for 3 days with conditioned medium collected from PA-317 packaging cells stably transfected with pCMV (□) or pCMV-dn3 (■). The infected BT-474, MDA-MB-453 and SK-BR-3 cells were then incubated with fresh medium for 2 days and then selected on G418 for a month before counting colonies. The mean average and range of duplicate 60 mm plates are shown.

Fig. 5. *Expression of dominant negative HER-3 in cells infected with pCMV-dn3.* Bright field microscopy of anti-HER-3 immunocytochemistry for BT-474 cells infected with pCMV (A) and BT-474 cells infected with pCMV-dn3 (B). The H105 antibody is known to be highly specific for HER-3 and binds to an epitope within the extracellular domain. While control cells express wild-type HER-3, the levels are low with immunodetection and HRP/DAB detection. Therefore, the high levels of HER-3 measured in BT-474 cells infected with pCMV-dn3 confirmed the ectopic expression of dominant negative HER-3 in these cells.

KEY RESEARCH ACCOMPLISHMENTS

- The 21MT-1-derived cell lines were used for additional studies of the effects of dominant negative HER-3 on HER-2/HER-3 activation, signaling and growth factor responses in culture and *in vivo*.

- BT-474, MDA-MB-453 and SK-BR-3 cells were all used to derive a set of new breast cancer cell lines with HER-2 gene amplification that also express dominant negative HER-3.

- The newly derived breast cancer cell lines are being used for studies of the effects of dominant negative HER-3 on HER-2/HER-3 activation, signaling and growth responses in culture and *in vivo*.

REPORTABLE OUTCOMES

A manuscript describing most of the work done with the H16N-2 and 21MT-1 cell lines was published shortly before the grant started (45). Another manuscript containing additional data and work using the BT-474, MDA-MB-453 and SK-BR-3 cell lines is presently in preparation.

CONCLUSIONS

Amplification and overexpression of the HER-2 gene is involved in the oncogenic transformation of mammary epithelial cells in approximately a third of breast cancer patients. In those individuals with HER-2 gene amplification, this dominant genetic event is likely to be the principle change which drives malignancy because HER-2 is such a potent oncogene when highly overexpressed in experimental systems. However, our understanding is still fragmentary concerning the exact mechanisms by which signals for cell growth are constitutively activated in breast cancer cells with HER-2 gene amplification, and methods for permanently inhibiting the constitutive activation of signaling in such cells have not yet met with great success. Our previous work showed that the high-level overexpression of HER-2 in 21MT-1 cells was associated with the constitutive activation of PI 3-kinase and growth factor independence in culture (22). By constitutively activating key mitogenic signaling pathways to a level that is effective for autonomous growth, tumor cells escape the normal controls on cell cycle regulation. Therefore, we sought to experimentally assess the importance of the cooperative interactions between HER-2 and HER-3 during the growth factor-independent proliferation of breast cancer cells with HER-2 gene amplification, as well as in cells stimulated by exogenous HRG. Our data now confirm the importance of the HER-2/HER-3 heterodimer interaction for receptor activation and the recruiting of key signaling molecules in breast cancer cells with HER-2 gene amplification, as well as in normal cells stimulated by HRG (45). Dominant negative HER-3 also potently inhibited the growth factor-independent and anchorage-independent growth of the 21MT-1 cells in culture (45). Therefore, our work studying the interaction between HER-2 and HER-3 offers exciting new opportunities for blocking the mechanism of autonomous growth in breast cancer cells with HER-2 gene amplification.

By itself, HER-3 is known to be almost completely kinase deficient (as would be expected from sequence analysis showing deleterious substitutions in the enzymatic site) and is not able to activate signaling in-and-of-itself in genetically engineered cell lines that do not co-express any of the other HER kinases. However, while the other HERs have active kinase domains, HER-3 contains multiple additional docking sites for $p85^{PI\ 3\text{-}kinase}$ and SHC proteins not found in the other HERs. Also, as mentioned above, HER-2 is known to be an especially active tyrosine kinase that exhibits ligand-independent activation when overexpressed. These combined considerations (i.e. HER-3 docking sites combined with HER-2 kinase potential) may account for the especially potent activation of signaling induced by HER-2/HER-3 heterodimers in response to HRG in H16N-2 cells (22, 45, 46), and that is constitutively activated in 21MT-1 cells (22, 45). Interestingly, the blocking of HER-2/HER-3 function with dominant negative HER-3 showed specificity in that the H16N-2 and 21MT-1 cells infected with pCMV-dn3 and selected on G418 still proliferated in response to exogenous EGF. This suggests that HER-1/HER-3 was not as effectively inhibited by dominant negative HER-3 in these cells as was HER-2/HER-3. As mentioned above, HER-1 and HER-3 interact to some extent in these (46) and other cell lines (34, 35), and this interaction may be required for EGF-stimulated growth (46). However, the relative affinity of HER-1/HER-3 heterodimers is very weak compared to HER-2/HER-3 heterodimers when compared with cross-linking analysis (42-44). Therefore, in order to further investigate the preferential effect that dominant negative HER-3 had on HER-2/HER-3-mediated growth, we also compared the effects of dominant negative HER-3 on both HRG- and EGF-induced activation of HERs with anti-phosphotyrosine immunoblotting (Fig. 3). While dominant

negative HER-3 inhibited the levels of tyrosine phosphorylated HER-2/HER-3 in 21MT-1-dn3 cells, the EGF-induced tyrosine phosphorylation of EGFR-containing dimers was not apparently affected by dominant negative HER-3 in cells under these conditions (Fig.3). This result is also consistent with the hypothesis that EGFR-containing dimers are not as effectively inhibited by dominant negative HER-3 as are HER-2/HER-3 heterodimers for a given level of dominant negative HER-3 expressed in genetically engineered cell lines.

We have now also generated BT-474, MDA-MB-453 and SK-BR-3 cells stably infected with either pCMV or pCMV-dn3, and have confirmed that the cells infected with pCMV-dn3 express dominant negative HER-3 (Fig. 5). We are presently using these cell lines to further study the effects of dominant negative HER-3 on HER-2/HER-3 activation, signaling and growth responses as originally discussed in the grant proposal. While we have generating BT-474, MDA-MB-453 and SK-BR-3 cells infected with these vectors, we have also seen a much lower number of colonies growing out during selection on G418 for cells infected with the pCMV-dn3 than for those infected with pCMV (Fig. 4). Furthermore, we noticed that many of the colonies that start to grow out in plates infected with pCMV-dn3 do not continue to grow, suggesting that the growth of many of the cell clones infected with pCMV-dn3 are inhibited by the expression of dominant negative HER-3. This is also consistent with the heterogeneity in anti-HER-3 staining seen for cells infected with pCMV-dn3 (Fig. 5B). Therefore, the low number of colonies seen for cells infected with pCMV-dn3 may indicate that the growth of many of the cells which express the highest levels of dominant negative HER-3 is inhibited, and thus only form attenuated colonies. We are also planning to isolate individual cell clones for further analysis.

In contrast to the 21MT-1 cells, little is known about the growth factor requirements for BT-474, MDA-MB-453, and SK-BR-3 cells, which are all usually cultured in medium containing 10% fetal calf serum. Therefore, it may be difficult to maintain cell populations that express the higher levels of dominant negative HER-3 under conditions where constitutive and regulative growth responses have not yet been determined as for the 21MT-1 cells. If the highest level expressing cells are being selected out because of the inhibitory effects of dominant negative HER-3, this may necessitate the use of an inducible expression system to directly study the full extent of the effects of dominant negative HER-3 in these cells. Fortunately, we have located the ideal expression vector that will allow us to construct cell lines that express inducible dominant negative HER-3. The tetracycline-inducible pRevTet-Off and pRevTRE are retroviral expression vectors that facilitate the inducible expression of ectopic genes in mammalian cells (Clontech-see Appendices). Therefore, we are planning to construct an inducible dominant negative HER-3 vector using pRevTRE, which contains Sal I and Cla I sites that can be used to insert the same Sal I-Cla I dominant negative HER-3 fragment that was used to construct pCMV-dn3 (45). The newly constructed vector will then be used to perform infections of BT-474, MDA-MB-453 and SK-BR-3 cells after being infected with pRevTet-Off and selected on G418. The pRevTRE vector utilizes a hygromycin resistance gene, and double selection on G418 and hygromycin will then allow use to derive cell lines that express the Tetracycline transcriptional regulator protein and also contain the dominant negative HER-3 gene driven by the CMV-TRE promoter. The cells infected with the inducible form of dominant negative HER-3 will also be cultured in the presence of tetracycline to keep the dominant negative HER-3 gene off during the selection on hygromycin. Subsequent to selection on hygromycin, cells with (as a control) and without tetracycline will then be used for experiments as we have outlined in the proposal to determine the effectiveness of dominant negative HER-3 for inhibiting HER-2/HER-3 activation, signaling and

growth responses in culture and *in vivo*. This "Tet-Off" system will also be ideal in that we can maintain the cells in the presence of tetracycline prior to injection into mice, and with removal of tetracycline the cells will then turn on the dominant negative HER-3 gene at the start of the experiment. This system also allows a wide range of induction through the TRE element depending on the concentration of tetracycline employed. This will also be very useful for directly studying the stoichiometric aspect of receptor inhibition by using various concentrations of Tetracycline in culture experiments.

In summary, we have used the pCMV-dn3 vector to inactivate the function of HER-2/HER-3 in breast cancer cells with HER-2 gene amplification. The inhibition of HERs by dominant negative HER-3 shows selectivity by preferentially inhibiting HER-2/HER3 function and growth responses involving HER-2/.HER-3. We are continuing to develop a number of cell lines to study the relative importance of the HER-2/HER-3 interaction for activating cell signaling and uncontrolled growth in breast cancer cells with HER-2 gene amplification.

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APPENDICES

1) Copy of Ram et al. 2000, Cell Growth & Diff. 11:173-83.
2) Copy of Ram et al. 2000, J. Cell. Phys., 183:301-13.
3) Information on the pRevTRE retroviral expression vector (Clontech).

Blocking HER-2/HER-3 Function with a Dominant Negative Form of HER-3 in Cells Stimulated by Heregulin and in Breast Cancer Cells with HER-2 Gene Amplification[1]

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Abstract

Amplification and overexpression of the HER-2 (*neu*/*erb*B-2) gene in human breast cancer are clearly important events that lead to the transformation of mammary epithelial cells in approximately one-third of breast cancer patients. Heterodimer interactions between HER-2 and HER-3 (*erb*B-3) are activated by *neu* differentiation factor/heregulin (HRG), and HER-2/HER-3 heterodimers are constitutively activated in breast cancer cells with HER-2 gene amplification. This indicates that inhibition of HER-2/HER-3 heterodimer function may be an especially effective and unique strategy for blocking the HER-2-mediated transformation of breast cancer cells. Therefore, we constructed a bicistronic retroviral expression vector (pCMV-dn3) containing a dominant negative form of HER-3 in which most of the cytoplasmic domain was removed for introduction into cells. By using a bicistronic retroviral vector in which the antibiotic resistance gene and the gene of interest are driven by a single promoter, we attained 100% coordinate coexpression of antibiotic resistance with the gene of interest in target cell populations. Breast carcinoma cells with HER-2 gene amplification (21 MT-1 cells) and normal mammary epithelial cells without HER-2 gene amplification from the same patient (H16N-2 cells) were infected with pCMV-dn3 and assessed for HER-2/HER-3 receptor tyrosine phosphorylation, $p85^{PI\ 3\text{-}kinase}$ and SHC protein activation, growth factor-dependent and -independent proliferation, and transformed growth in culture. Dominant negative HER-3 inhibited the HRG-induced activation of HER-2/HER-3 and signaling in H16N-2 and 21 MT-1 cells as well as the constitutive activation of HER-2/HER-3 and signaling in 21 MT-1 cells. Responses to exogenous HRG were strongly inhibited by dominant negative HER-3. In contrast, the proliferation of cells stimulated by epidermal growth factor was not apparently affected by dominant negative HER-3. The growth factor-independent proliferation and transformed growth of 21 MT-1 cells were also strongly inhibited by dominant negative HER-3 in anchorage-dependent and -independent growth assays in culture. Furthermore, the HRG-induced or growth factor-independent proliferation of 21 MT-1 cells was inhibited by dominant negative HER-3, whereas the epidermal growth factor-induced proliferation of these cells was not: this indicates that dominant negative HER-3 preferentially inhibits proliferation induced by HER-2/HER-3.

Introduction

The HER-2 (*neu*/*erb*B-2) gene encodes a M_r 185,000 protein tyrosine kinase that is highly homologous to the EGF[3] receptor (HER-1/EGFR/*erb*B-1), HER-3 (*erb*B-3), and HER-4 (*erb*B-4; Refs. 1–3), which together comprise the type 1 family of receptor tyrosine kinases (4, 5). The HER family receptor tyrosine kinases all contain ectodomains with two cysteine-rich sequences. Despite this structural homology, these receptors differ in their ligand specificities (4). Thus, HER-1 binds several ligands closely related to EGF, whereas HER-3 and HER-4 are the receptors for a number of different isoforms of *neu* differentiation factor/HRG (6–8). Whereas no direct ligand for HER-2 has yet been cloned, it is now clear that HER-2 is capable of heterodimerization with HER-1 (9, 10), HER-3 (11, 12), or HER-4 (8), and these HER-2-containing heterodimers form the highest affinity binding sites for their respective ligands (10, 11). HER-2 is amplified in 28% of primary breast carcinomas *in vivo* (13), and another 10% of primary breast carcinomas overexpress HER-2 without amplification of the gene (14–16). In addition, HER-2 gene amplification concordant with high-level overexpression is associated with increased tumor aggressiveness and the poor prognosis of breast cancer patients (13, 14, 17–19). Other related genes, such as the HER-1 gene, are sometimes amplified in human breast cancers (13). However, amplification of the HER-1 gene is much less common than that seen for HER-2 (2% *versus* 28%, respectively) in breast cancer. Whereas amplification of HER-3 or HER-4 has not been seen in various studies (2, 3), our own work and the studies of

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[3] The abbreviations used are: EGF, epidermal growth factor; BSA, bovine serum albumin; HRG, *neu* differentiation factor/heregulin; IGF, insulin-like growth factor; PI 3-kinase, phosphatidylinositol 3-kinase; SF, serum-free; TBS, Tris-buffered saline; TTBS, Tween TBS; HRP, horseradish peroxidase; DAB, diaminobenzidine; TTBS, 150 mM NaCl, 50 mM Tris (pH 7.5), and 0.1% Tween 20.

others (20–23) have now shown that heterodimer interactions between HER-2 and HER-3 are constitutively activated in breast cancer cells with HER-2 gene amplification, and cotransfection of HER-3 with HER-2 greatly augments the transforming capability of HER-2 in genetically engineered cell lines (21). HER-2/HER-3 heterodimer complexes are now thought to potently activate the PI 3-kinase and mitogen-activated protein kinase signal transduction pathways to a level that is effective for transformation. We are particularly interested in how the cooperative effects of HER-2 and HER-3 activate various mitogenic signal transduction pathways involved in cell growth.

Experimentally elevated HER-2 gene expression in various cell lines, including nontransformed human mammary epithelial cells, induces the complete transformation of cells injected into nude mice (24–27). The potent oncogenic potential of HER-2 is generally thought to be due to its ability to constitutively activate various key signal transduction pathways that are involved in the regulation of cell growth. However, whereas our current understanding of the oncogenic potential of HER-2 has expanded quite rapidly (for review, see Ref. 28), our knowledge of exactly how HER-2 induces the neoplastic transformation of human mammary epithelial cells still remains fragmentary. For example, although HER-2 was originally discovered as the *neu* transmembrane-mutated form of the gene in rat neuroblastoma cells (29), the HER-2 gene found in human breast cancer has never shown such mutations (30), but the level of tyrosine-phosphorylated HER-2 in primary human breast cancer *in vivo* always shows a direct correspondence with the overexpression of HER-2 (31). This suggests that high-level overexpression of wild-type HER-2 alone is sufficient to constitutively activate its tyrosine kinase function. Furthermore, the protein encoded by the wild-type HER-2 gene was also previously shown to possess constitutive tyrosine kinase activity if sufficiently overexpressed in a variety of cell lines in culture in the absence of any identifiable ligand (24–27, 32, 33), and transfection of a gene encoding a chimeric receptor containing the HER-1 extracellular domain fused to the cytoplasmic domain of HER-2 results in the constitutive tyrosine kinase activity of the chimeric receptor in the absence of EGF (32, 33). This indicates that the tyrosine kinase domain of HER-2 exhibits a greater tendency toward ligand-independent activation than do the other HERs when overexpressed.

Another area of great importance concerns the heterodimeric associations that are now known to occur between the different HER proteins, including HER-1 and HER-2 (9, 10), HER-2 and HER-3 (11, 12), HER-2 and HER-4 (8), and HER-1 and HER-3 (34, 35) in response to ligands. Our own work and that of others (20–22) has now established that the heterodimer interactions between HER-2 and HER-3 are also constitutively activated in breast cancer cells with HER-2 gene amplification, and the cooperative interactions between HER-2 and HER-3 are associated with the constitutive activation of various signaling pathways in cancer cells with HER-2 gene amplification. However, the involvement of HER-2/HER-3 heterodimers in the constitutive activation of signaling pathways that transform cancer cells with HER-2 gene amplification has not yet been tested with perturbative analysis. One strategy that has been used successfully to block the function of other receptor tyrosine kinases uses dominant negative expression vectors in which the region coding for the cytoplasmic domain of the receptor is almost completely removed. Although the truncated receptor still contains the transmembrane domain and can thus dimerize within the cell, it lacks tyrosine kinase activity and inhibits the signal transduction docking function. This strategy has been used effectively to block HER-1 (36), platelet-derived growth factor receptor (37), and fibroblast growth factor receptor (38). Recently, a dominant negative HER-2 vector was also used successfully to block HER-2 function in normal mouse development (39). The use of such HER-2 vectors has apparently not yet been useful for blocking HER-2 function in cancer cells with HER-2 gene amplification, probably due to the stoichiometric problems associated with the inability to generate mutant/wild-type levels high enough for effective inhibition. However, the fact that HER-3 is not highly overexpressed in these cells and that activated HER-2 and HER-3 have a particularly high affinity interaction (40–42) suggests that dominant negative HER-3 may be especially effective in blocking HER-2/HER-3 function.

Results

H16N-2 and 21 MT-1 Cells Provide a Model System for Studying the Role of HER-2/HER-3 in HRG-induced Mitogenesis and the Autonomous Growth of Cancer Cells with HER-2 Gene Amplification. For these studies, we used cell lines originally isolated from a patient with infiltrating and intraductal carcinoma of the breast with HER-2 gene amplification (43, 44). The 21 MT-1 metastatic breast carcinoma cell line was isolated from a pleural effusion collected during an advanced stage of the disease. H16N-2 cells are nonneoplastic cells isolated from normal mammary tissue of the same patient; thus, they serve as an ideal control for studying the 21 MT-1 cells as well as the effects of HRG in nontransformed cells. RFLP analysis had previously shown that these cell lines share common genetic polymorphisms (43), and we have also verified that the H16N-2 and 21 MT-1 cells are derived from a single individual by DNA fingerprinting analysis of a hypervariable region of the BRCA-1 locus.[4] We have previously shown that the amplification and high-level overexpression of HER-2 in the 21 MT-1 cells is associated with HER-2/HER-3-mediated activation of PI 3-kinase and growth factor independence (*i.e.*, autonomous growth) in SF culture (22, 45). This system is ideal for studying receptor activation and signaling under well-defined conditions that allow us to distinguish constitutive from externally mediated growth factor responses in culture. To directly measure the activation of HER-2 and HER-3 in these cells, we starved the cells of growth factors for 48 h in SF medium and then directly extracted the cells (Fig. 1, *A* and *B*, *Lanes 1* and *3*) or stimulated the cells with HRG-β for 10 min before extraction (Fig. 1, *A* and *B*, *Lanes 2* and *4*). Immunoprecipitation followed by immunoblotting directly showed the levels of

[4] Unpublished observations.

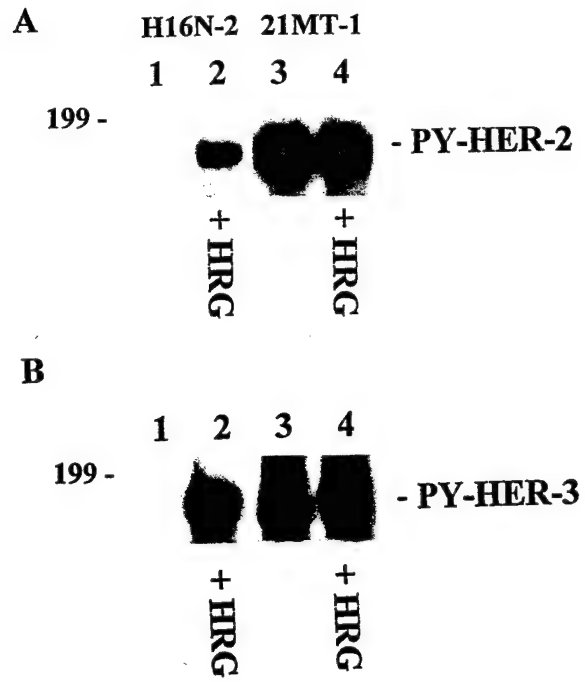

Fig. 1. Activation of HER-2/HER-3 by HRG and its constitutive activation in breast cancer cells with HER-2 gene amplification. Samples containing 2 mg of cell lysate protein were immunoprecipitated with anti-phosphotyrosine antibody, followed by immunoblotting with anti-HER-2 antibody (*A*) or immunoprecipitation with anti-HER-3 antibody followed by immunoblotting with anti-phosphotyrosine antibody (*B*) to show the level of HER-2 and HER-3 activated in cells with or without HRG stimulation in culture. H16N-2 nontransformed human breast epithelial cells (*Lanes 1* and *2*) and 21 MT-1 metastatic breast carcinoma cells with HER-2 gene amplification (*Lanes 3* and *4*) were starved of growth factors for 48 h in SF medium and then directly extracted (*Lanes 1* and *3*) or stimulated with 10 ng/ml HRG-β for 10 min at 37°C before extraction (*Lanes 2* and *4*).

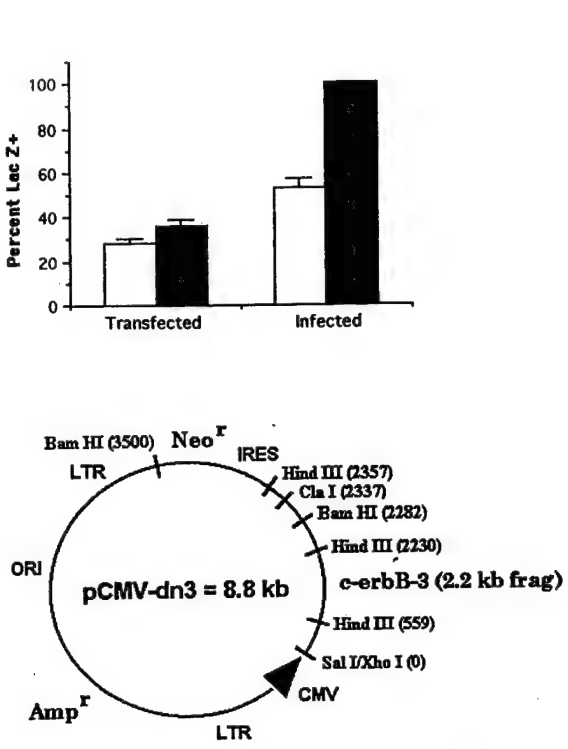

Fig. 2. Development of the dominant negative HER-3 bicistronic retroviral expression vector. *A*, to test for the efficiency of antibiotic resistance gene and marker gene coexpression in our cell lines, both monocistronic (double promoter) and bicistronic (single promoter) forms of a retroviral expression vector containing the Neo^r and LacZ$^+$ genes were either transfected into or used to infect H16N-2 cells. Colonies selected on G418 for a month were then stained for β-galactosidase activity and counted to determine the percentage of blue-stained colonies. The mean average ± SD for triplicate wells is shown. □, monocistronic; ■, bicistronic. *B*, the pCMV-dn3 bicistronic retroviral expression vector was constructed containing a dominant negative form of the HER-3 gene in which most of the cytoplasmic domain of HER-3 was removed. This vector also contains an internal ribosome-binding site (*IRES*) between the HER-3 gene and the Neo^r gene located downstream, which together form a single transcription unit. The expression of the pCMV-dn3 bicistronic transcript in mammalian cells then allows for the efficient coordinate coexpression of the dominant negative HER-3 gene with antibiotic resistance in retroviral-infected cell populations.

HER-2 and HER-3 activated in these cells. HRG induced tyrosine phosphorylation of both HER-2 and HER-3 in H16N-2 cells (Fig. 1, *A* and *B, Lane 2*), whereas 21 MT-1 cells show high-level constitutive activation of both HER-2 and HER-3 in the absence of exogenous growth factors in culture (Fig. 1, *A* and *B, Lane 3*) due to the amplification and overexpression of HER-2 in these cells (22, 45).

Construction of H16N-2 and 21 MT-1 Cell Lines Expressing Dominant Negative HER-3. We previously found that the introduction of standard monocistronic expression vectors (in which the antibiotic resistance gene and marker gene are driven by separate promoters) into many different cell lines did not lead to very efficient coordinate coexpression of antibiotic resistance with the gene of interest. Experiments were performed using retroviral control vectors containing the Neo^r and LacZ$^+$ genes placed in either monocistronic or bicistronic configuration and then either transfected or infected into target cell lines to assess the LacZ$^+$ gene expression in G418-selected cell colonies (Fig. 2*A*). The results showed that cells infected with the bicistronic retroviral vector (in which the LacZ$^+$ and Neo^r genes form a single transcription unit driven by one promoter) coordinately coexpressed antibiotic resistance with LacZ$^+$ expression in 100% of the G418-selected cell colonies. Thus, the infection of bicistronic retroviral vectors completely eliminated the occurrence of false positive clones in genetically engineered cells and resulted in greater efficiency of LacZ$^+$ gene expression within cell clones as well (data not shown). These results attained using the H16N-2 cells are also similar to those seen for a number of different mammary epithelial cell lines, including the 21 MT-1 cells (data not shown). Therefore, we used the pCMV bicistronic retroviral vector to express dominant negative HER-3 in target cells. By using the pBK-CMV phagemid expression vector (Stratagene) as an intermediate, we cloned a dominant negative HER-3 fragment into the pCMV bicistronic retroviral expression vector using flanking restriction sites located within the extensive polylinker region of pBK-CMV. The human HER-3 cDNA was used to clone a 2.2-kb fragment of HER-3 lacking most of the cytoplasmic domain into pBK-CMV to generate pBK-CMV-dn3, and this ligation also introduced an in-frame stop codon

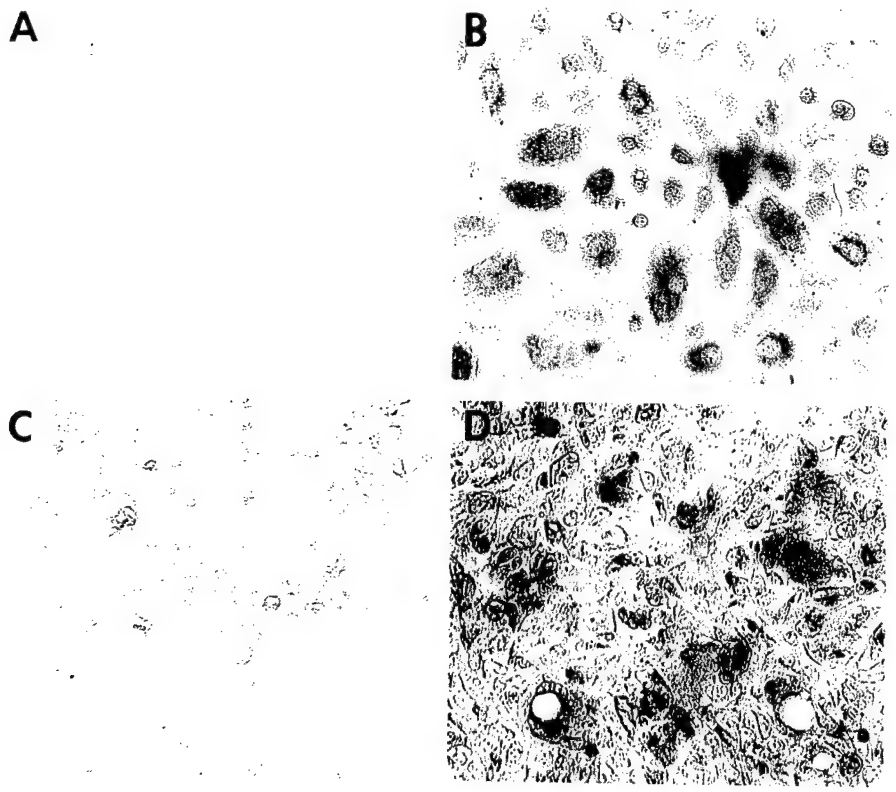

Fig. 3. Expression of dominant negative HER-3 in cells infected with pCMV-dn3. Immunocytochemistry with anti-HER-3 monoclonal antibody was used to measure the level of HER-3 protein in H16N-2 cells (*A*), H16N-2-dn3 cells (*B*), 21 MT-1 cells (*C*), and 21 MT-1-dn3 cells (*D*). The H105 antibody is known to be highly specific for HER-3 and binds to an epitope within the extracellular domain. Although control cells express wild-type HER-3 (Fig. 6), the levels are below the level for immunodetection with HRP/DAB staining. Therefore, the easily detectable levels of HER-3 measured in H16N-2-dn3 and 21 MT-1-dn3 cells confirmed the ectopic expression of dominant negative HER-3 in cells infected with pCMV-dn3.

12 codons downstream of the point of ligation. The dominant negative HER-3 insert removed from pBK-CMV-dn3 was then cloned into pCMV to generate pCMV-dn3 (Fig. 2*B*). Restriction digest analysis confirmed the proper construction of the vectors (data not shown). H16N-2 and 21 MT-1 cells were then infected with the pCMV backbone (used as a control) or pCMV-dn3 using the ψCRIP packaging cell line. The cell lines infected with the control vector are referred to as H16N-2 and 21 MT-1 cells, whereas those infected with pCMV-dn3 are referred to as H16N-2-dn3 and 21 MT-1-dn3 cells. Immunocytochemistry was performed to confirm that the H16N-2-dn3 and 21 MT-1-dn3 cells express the dominant negative HER-3 (Fig. 3). Expression of the ectopic HER-3 protein was assessed using the H105 anti-HER-3 monoclonal antibody that binds specifically to an epitope within the extracellular domain of HER-3. Although H16N-2 and 21 MT-1 cells express wild-type HER-3, the wild-type HER-3 protein levels are below the level for immunodetection with HRP/DAB staining using immunocytochemistry (Fig. 3, *A* and *C*). Therefore, the easily detectable levels of HER-3 measured in H16N-2-dn3 and 21 MT-1-dn3 cells (Fig. 3, *B* and *D*) readily confirmed the expression of dominant negative HER-3 in cells infected with pCMV-dn3.

Inhibition of HER-2/HER-3 Activation in Cells Expressing Dominant Negative HER-3. We next measured the effects of dominant negative HER-3 on the activation of HER-2/HER-3 in anti-phosphotyrosine immunoblots (Fig. 4*A*). Dominant negative HER-3 potently inhibited the HRG-induced tyrosine phosphorylation of HER-2/HER-3 in H16N-2-dn3 and 21 MT-1-dn3 cells (Fig. 4*A*, *Lanes 4* and *8*) as well as the constitutive tyrosine phosphorylation of HER-2/HER-3 in the 21 MT-1-dn3 cells (Fig. 4*A*, *Lane 7*). We also separately measured the levels of tyrosine-phosphorylated HER-2 and HER-3 by immunoprecipitation followed by immunoblotting (Fig. 5), which showed that dominant negative HER-3 inhibited HER-2 recruitment in anti-phosphotyrosine immunoprecipitates (Fig. 5*A*) and almost completely blocked HER-3 tyrosine phosphorylation (Fig. 5*B*) in H16N-2-dn3 and 21 MT-1-dn3 cells. Furthermore, immunoblots probed for HER-2 or HER-3 showed no significant effect of dominant negative HER-3 on the level of the wild-type HER-2 or HER-3 in H16N-2-dn3 and 21 MT-1-dn3 cells (Fig. 6), indicating that the effects of dominant negative HER-3 in H16N-2-dn3 and 21 MT-1-dn3 cells do not involve other effects on the expression of wild-type HER-2 or HER-3.

Inhibition of HER-2/HER-3-mediated Signaling in Cells Expressing Dominant Negative HER-3. We next measured the effects of dominant negative HER-3 on the recruitment and tyrosine phosphorylation of the signaling molecules, $p85^{PI\ 3\text{-}kinase}$, $p46^{SHC}$, and $p52^{SHC}$ in cells with and without HRG stimulation in culture. The dominant negative HER-3 was found to inhibit the recruitment of $p85^{PI\ 3\text{-}kinase}$ in anti-phosphotyrosine immunoprecipitates (Fig. 7). We have previously shown that this assay is a very reliable measure of the recruitment and activation of $p85^{PI\ 3\text{-}kinase}$ by HER-2/HER-3 (22). We (22) and others (46–48) have also found previously that activation of PI 3-kinase by various receptor tyrosine kinases involves recruitment of PI 3-kinase but does not involve detectable tyrosine phosphorylation of $p85^{PI\ 3\text{-}kinase}$ under more physiological conditions where $p85^{PI\ 3\text{-}kinase}$ is not artificially overexpressed (46).

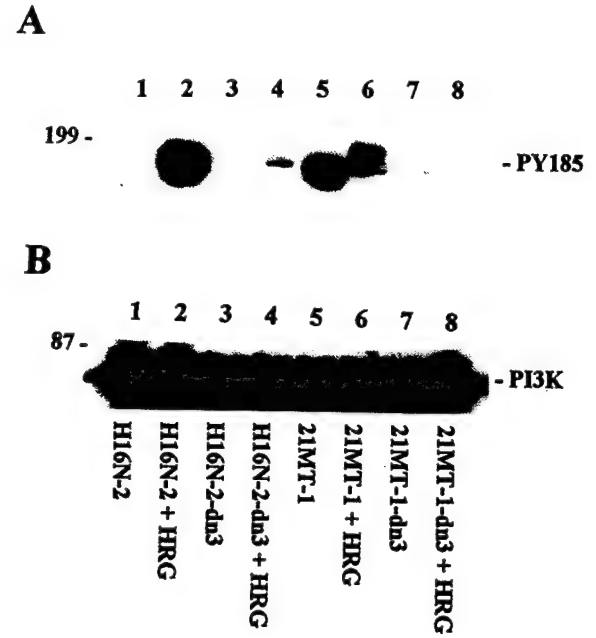

Fig. 4. Inhibition of HER-2 and HER-3 activation in cells expressing dominant negative HER-3. *A*, samples containing 100 μg of cell lysate protein were immunoblotted with anti-phosphotyrosine antibody. *B*, the same blot was then reprobed with anti-p85 antibody as a control to confirm equal loading of the gel. H16N-2 cells (*Lanes 1* and *2*), H16N-2-dn3 cells (*Lanes 3* and *4*), 21 MT-1 cells (*Lanes 5* and *6*), and 21 MT-1-dn3 cells (*Lanes 7* and *8*) were starved of growth factors for 48 h in serum-free medium and then directly extracted (*Lanes 1, 3, 5,* and *7*) or stimulated with HRG-β before extraction (*Lanes 2, 4, 6,* and *8*).

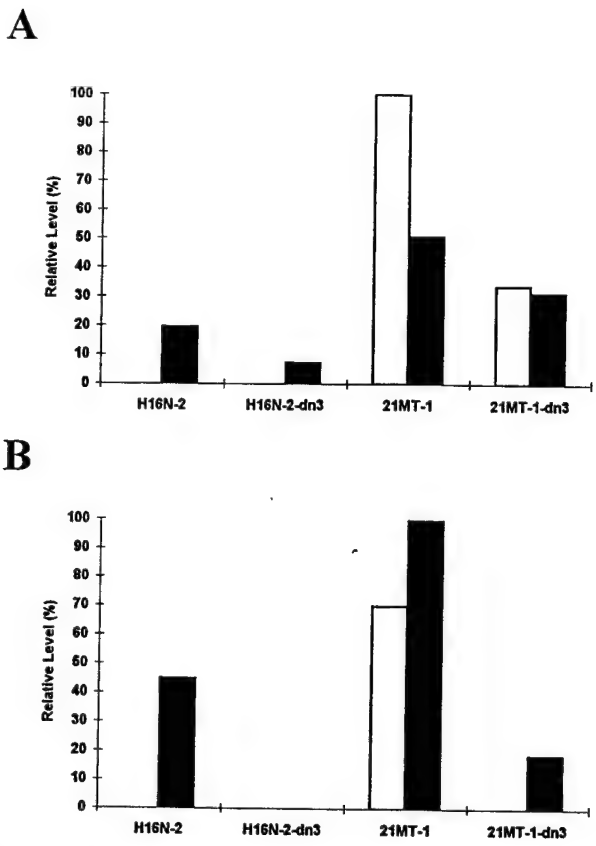

Fig. 5. Dominant negative HER-3 inhibition of HER-2 and HER-3 tyrosine phosphorylation. Immunoprecipitation followed by immunoblotting was used to separately determine the levels of tyrosine-phosphorylated HER-2 (*A*) and HER-3 (*B*) in the different cell lines with or without stimulation with HRG-β performed exactly as that shown in Fig. 1. The results shown here were attained from scanning densitometry of negatives exposed by chemiluminescent substrate. □, SF; ■, SF + HRG.

Therefore, the changes measured in the recruitment of $p85^{PI\ 3\text{-}kinase}$ in anti-phosphotyrosine immunoprecipitates reflect the level of PI 3-kinase recruited by activated receptor complexes (22). Furthermore, dominant negative HER-3 inhibited the recruitment of $p46^{SHC}$ and $p52^{SHC}$ in anti-phosphotyrosine immunoprecipitates (Fig. 8). However, in the case of SHC proteins, which are known to be highly tyrosine-phosphorylated during activation, the level in anti-phosphotyrosine immunoprecipitates likely reflects the combined effects on the tyrosine phosphorylation of SHC proteins as well as the level recruited by activated receptor complexes. In summary, the cells expressing dominant negative HER-3 showed impaired HER-2/HER-3 function as well as significant reductions in the recruitment and tyrosine phosphorylation of signaling molecules for both the PI 3-kinase and mitogen-activated protein kinase signal transduction pathways.

Dominant Negative HER-3 Inhibits HRG-induced Proliferation and the Autonomous Growth of Breast Cancer Cells with HER-2 Gene Amplification. We routinely use the H16N-2 and 21 MT-1 cell lines for our studies because they were derived from the same patient and can be grown under completely defined SF conditions in culture. This well-defined system allows us to study growth factor responses as well as growth factor-independent (*i.e.*, autonomous) proliferation in culture in a manner that is not possible for other cell lines derived in high serum-containing conditions. Anchorage-dependent monolayer growth assays with and without exogenous growth factors showed that dominant negative HER-3 inhibited the HRG-induced proliferation of both H16N-2-dn3 and 21 MT-1-dn3 cells in culture (Fig. 10*A*). In contrast, dominant negative HER-3 had no apparent effect on the insulin/EGF-induced proliferation of H16N-2-dn3 and 21 MT-1-dn3 cells in culture (Fig. 10*A*). Furthermore, the proliferation of the 21 MT-1-dn-3 cells was completely blocked in the absence of exogenous growth factors in culture (Figs. 9 and 10*A*). These results indicate that dominant negative HER-3 preferentially inhibits only proliferation induced by HRG or the growth factor-independent proliferation of cells that that overexpress HER-2. Finally, soft agarose growth assays were also performed to assess the potential effects of dominant negative HER-3 on the anchorage-independent growth of 21 MT-1-dn3 cells in culture. Dominant negative HER-3 strongly blocked the transformed growth of 21 MT-1-dn3 cells in soft agarose and inhibited growth even with maximal activation of HER-2/HER-3 in the presence of exogenous HRG (Fig. 10*B*).

Discussion

Amplification and overexpression of the HER-2 gene in human breast cancer are clearly important events that lead to

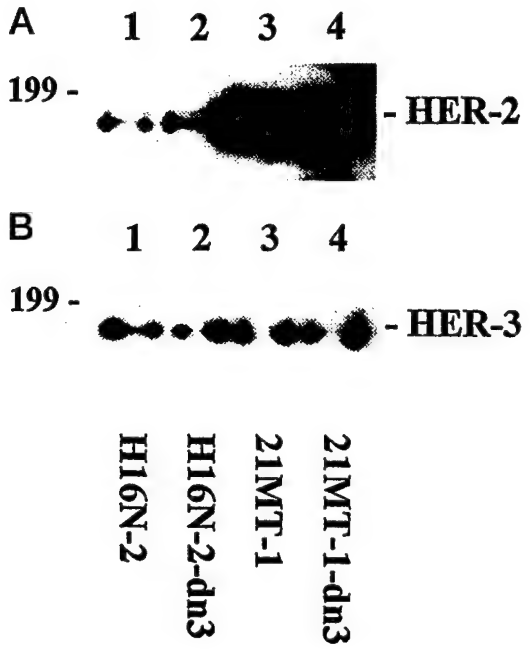

Fig. 6. Dominant negative HER-3 has no effect on the levels of endogenous HER-2 or HER-3. Samples containing 100 μg of cell lysate protein were immunoblotted with anti-HER-2 (*A*) or anti-HER-3 (*B*) antibody. H16N-2 cells (*Lane 1*), H16N-2-dn3 cells (*Lane 2*), 21 MT-1 cells (*Lane 3*), and 21 MT-1-dn3 cells (*Lane 4*) are shown.

the transformation of mammary epithelial cells in approximately one-third of breast cancer patients. In those individuals with HER-2 gene amplification, this dominant genetic event is likely the principle change that drives malignancy because HER-2 is such a potent oncogene when highly overexpressed in experimental systems. However, our understanding is still fragmentary concerning the exact mechanisms by which signals for cell growth are constitutively activated in breast cancer cells with HER-2 gene amplification, and methods for permanently inhibiting the constitutive activation of signaling in such cells have not yet met with great success. Our recent insights into the interaction between HER-2 and HER-3 offer exciting new opportunities for blocking the mechanism of autonomous growth in breast cancer cells with HER-2 gene amplification. By constructing cell lines that stably express a dominant negative form of HER-3, we have now successfully targeted the interaction between HER-2 and HER-3 in cells stimulated by exogenous HRG as well as that which is constitutively activated in breast cancer cells with HER-2 gene amplification. Also, the use of the pCMV-dn3 bicistronic retroviral vector results in highly efficient coordinate coexpression of antibiotic resistance and dominant negative HER-3 in target cell lines.

Our previous work showed that the elevated levels of HER-2 overexpression in 21 MT-1 cells are associated with high-level constitutive activation of PI 3-kinase and growth factor independence in culture (22, 45). In the present study, we sought to experimentally assess the importance of the cooperative interactions that occur between HER-2 and HER-3 in cells in response to HRG and during the growth factor-independent proliferation of breast cancer cells with HER-2 gene amplification. Our data now confirm the importance of the HER-2/HER-3 heterodimer interaction for recruiting key mitogenic signal transduction molecules involved in the growth of normal cells stimulated by HRG as well as in breast cancer cells with HER-2 gene amplification. Dominant negative HER-3 was able to block the HRG-induced proliferation of H16N-2 and 21 MT-1 cells as well as the growth factor-independent proliferation of 21 MT-1 cells in growth factor-free medium. In addition, dominant negative HER-3 potently inhibited the anchorage-independent growth of 21 MT-1 cells in soft agarose culture. These major effects of dominant negative HER-3 on cell proliferation do not necessarily preclude additional effects involving the rate of apoptosis in these cells, which remains to be determined. Also, preliminary *in vivo* studies have been performed using the 21 MT-1 and 21 MT-1-dn3 cells for injection into nude mice. However, to date, the 21 MT-1 control cell line has not been sufficiently tumorigenic in our nude mice to allow us to sufficiently test the effects of dominant negative HER-3 *in vivo*. The low tumor take and limited growth seen for 21 MT-1 cells in nude mice are apparently common problems for a significant number of highly malignant and metastatic breast carcinoma cell lines (49), and earlier studies with 21 MT-1 cells also suggested some difficulty in using these cells for tumor studies at later passages (50). Therefore, additional studies are under way using other breast carcinoma cell lines with HER-2 gene amplification as well as 21 MT-1 cells for transplantation into various immunodeficient mouse strains.

By itself, HER-3 is known to be almost completely kinase deficient (as would be expected from sequence analysis, which shows alterations in the enzymatic site) and is therefore unable to activate signaling in and of itself in genetically engineered cell lines that do not coexpress any of the other HER kinases (5, 40–42). However, whereas the other HERs have active kinase domains, HER-3 contains multiple additional docking sites for $p85^{PI\ 3\text{-}kinase}$ and SHC proteins not found in the other HERs (5). Also, as mentioned above, HER-2 is known to be an especially active tyrosine kinase that exhibits ligand-independent activation when overexpressed (33). These combined considerations (*i.e.*, HER-3 docking sites combined with HER-2 kinase potential) may account for the especially potent activation of signal transduction induced by HER-2/HER-3 heterodimers in response to HRG and seen constitutively in breast cancer cells (20–22). Interestingly, the blocking of HER-2/HER-3 function with dominant negative HER-3 was preferential in that the cells still proliferated in response to exogenous EGF, suggesting that the interaction between HER-1 and HER-3 is not necessary for mitogenesis in cells stimulated by EGF or that dominant negative HER-3 does not block HER-1/HER-3 function as well as HER-2/HER-3. In fact, it was this specificity of dominant negative HER-3 inhibition of HER-2/HER-3 that allowed us to use a constitutive promoter to express dominant negative HER-3, because the cells infected with pCMV-dn3 were still able to proliferate in response to EGF. Whereas there is evidence that HER-1 and HER-3 interact to some extent in these and other cell lines (34, 35, 51), the HER-1/HER-3 heterodimer interaction is clearly very weak compared to that for HER-2/HER-3 (40–42).

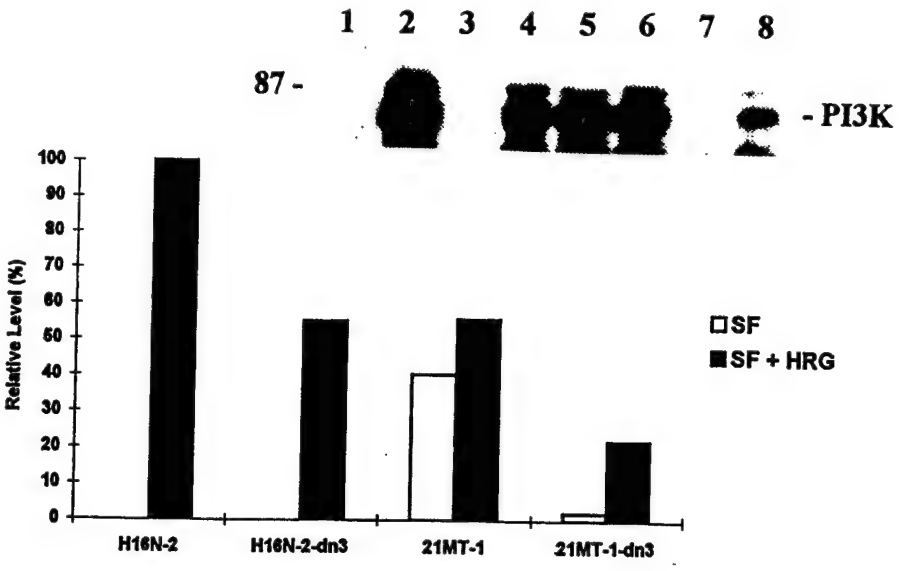

Fig. 7. Inhibition of PI 3-kinase recruitment by HER-2/HER-3 in cells expressing dominant negative HER-3. Samples containing 2 mg of cell lysate protein were immunoprecipitated with anti-phosphotyrosine antibody followed by immunoblotting with anti-p85 antiserum to show the level of $p85^{PI\ 3\text{-}kinase}$ recruited by tyrosine-phosphorylated receptor complexes. H16N-2 cells (*Lanes 1* and *2*), H16N-2-dn3 cells (*Lanes 3* and *4*), 21 MT-1 cells (*Lanes 5* and *6*), and 21 MT-1-dn3 cells (*Lanes 7* and *8*) were starved of growth factors for 48 h and then directly extracted (*Lanes 1, 3, 5,* and *7*) or stimulated with HRG-β before extraction (*Lanes 2, 4, 6,* and *8*).

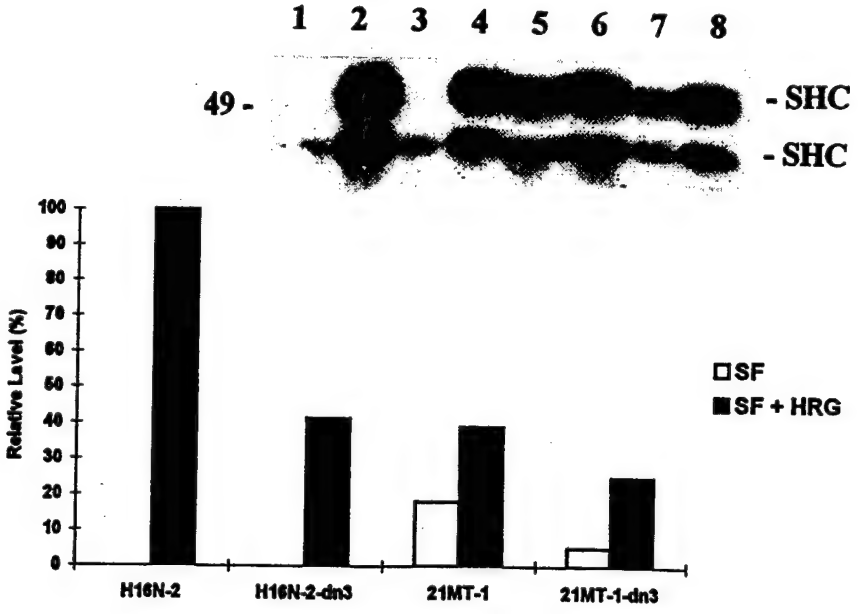

Fig. 8. Inhibition of SHC protein tyrosine phosphorylation and recruitment in cells expressing dominant negative HER-3. Samples containing 2 mg of cell lysate protein were immunoprecipitated with anti-phosphotyrosine antibody followed by immunoblotting with anti-SHC antiserum to show the level of $p46^{SHC}$ and $p52^{SHC}$ protein tyrosine phosphorylation and recruitment by tyrosine-phosphorylated receptor complexes. H16N-2 cells (*Lanes 1* and *2*), H16N-2-dn3 cells (*Lanes 3* and *4*), 21 MT-1 cells (*Lanes 5* and *6*), and 21 MT-1-dn3 cells (*Lanes 7* and *8*) were starved of growth factors for 48 h and then directly extracted (*Lanes 1, 3, 5,* and *7*) or stimulated with HRG-β before extraction (*Lanes 2, 4, 6,* and *8*).

Human breast carcinoma cells sometimes overexpress HER-3, and it has been suggested that this may be important for malignancy (2). However, whereas HER-3 is commonly expressed at a low but functional level in most nontransformed and transformed human mammary epithelial cells that we have tested, the HER-3 gene has never been found to be amplified or highly overexpressed, as is HER-2 (2). Furthermore, when cell lines are genetically engineered to overexpress HER-3, this alone is not sufficient to constitutively activate HER-3 or to transform cells (21). As mentioned above, HER-3 is a very weak kinase compared to the other HERs (5, 40–42), but HER-3 is constitutively activated in HER-2-overexpressing cell lines in which the cooperative interaction between HER-2 and HER-3 activates HER-2/HER-3 heterodimers (20–23). However, a number of the breast cancer cell lines with HER-2 gene amplification, such as 21 MT-1 cells, do not overexpress HER-3 in comparison with normal cells (Fig. 6). Therefore, it is our contention that low-level HER-3 cooperates with HER-2 to effectively transform breast carcinoma cells with HER-2 amplification, but this mechanism of cell transformation does not require concordant overexpression of HER-3.

Growth factor independence, as a phenotype, is a good indicator of progressive cell transformation in tumor cells with HER-2 gene amplification (22, 45). Normal human mammary epithelial cells require both IGF-I (or supraphysiological levels of insulin) and EGF to proliferate under

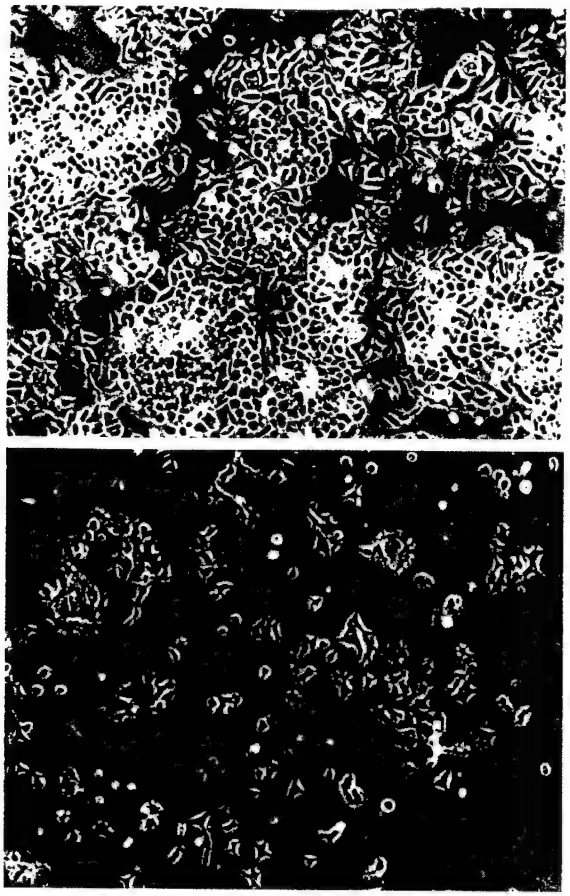

Fig. 9. Dominant negative HER-3 inhibits the autonomous proliferation of 21 MT-1 cells in monolayer culture. Phase-contrast microscopy of 21 MT-1 (*A*) and 21 MT-1-dn3 cells (*B*) cultured in SF medium in the complete absence of growth factors for 9 days is shown.

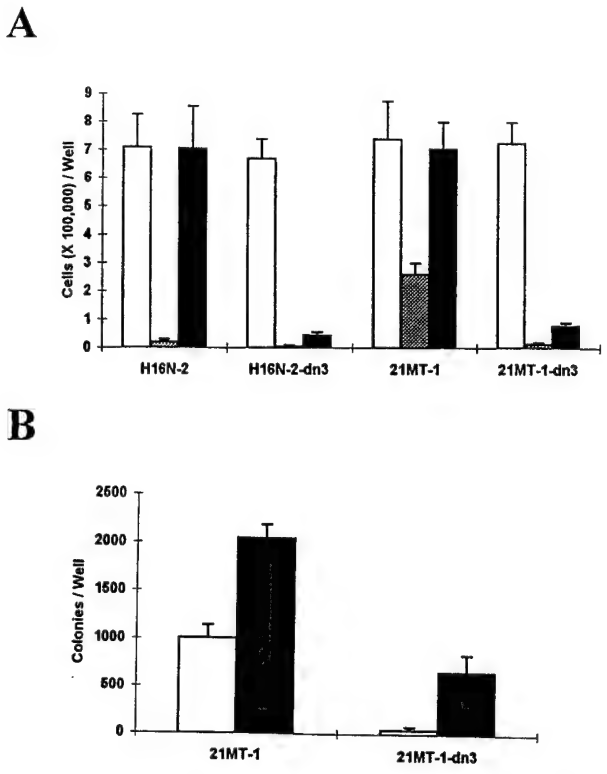

Fig. 10. Dominant negative HER-3 inhibits the HRG-induced proliferation of cells as well as the autonomous proliferation and transformed growth of 21 MT-1 cells in culture. *A*, anchorage-dependent growth assay showing the proliferation of H16N-2, H16N-2-dn3, 21 MT-1, and 21 MT-1-dn3 cells in monolayer culture for 9 days with SF medium plus insulin and EGF (□), without any growth factors (▨), or plus HRG-β (■). The mean average ± SD for triplicate wells is shown. *B*, anchorage-independent growth assay of 21 MT-1 and 21 MT-1-dn3 cells cultured for a month in soft agarose with or without HRG-β. The mean average ± SD for triplicate wells is shown. □, medium; ■, medium + HRG.

SF conditions in culture (52). The synergistic requirement for both IGF and EGF in the proliferation of normal mammary epithelial cells suggests that the attainment of growth factor-independent proliferation in mammary carcinoma cells involves genetic changes that subvert requirements for both IGF and EGF. We have previously shown that the 21 MT-2 and 21 MT-1 breast carcinoma cell lines have equivalently amplified HER-2 but show progressively elevated levels of HER-2 transcription associated with increasing IGF and EGF independence in culture (23, 45). We also found that HRG substitutes for both IGF and EGF in stimulating the proliferation of nontransformed human mammary epithelial cells (which express both HER-2 and HER-3, but not HER-4) in culture (45, 52). Therefore, we previously proposed that HER-2/HER-3 constitutive activation of signaling pathways in breast cancer cells substitutes for growth factor-mediated signaling, which usually requires the combination of IGF and EGF in normal cells (22, 45, 52). Furthermore, the distinguishing properties of HER-2/HER-3 function may help explain the occurrence, and potent oncogenicity and selection of amplified HER-2 in cell types that normally express HER-3.

Materials and Methods

Vector Construction. For experiments to test the efficiency of antibiotic resistance gene and marker gene coexpression in our cell lines, control retroviral expression vectors were constructed from the pCMV vector (originally derived from pSLH1001, which was derived from pLNCX) in which the Neo^r and the LacZ$^+$ genes were placed in either monocistronic or bicistronic configuration. The dominant negative HER-3 retroviral expression vectors were made using full-length human HER-3 cDNA (Amgen, Inc.), from which a 2.2-kb fragment missing most of the intracellular domain was generated by cutting out the insert with *Sal*I and *Bam*HI. By using the pBK-CMV phagemid expression vector (Stratagene) as an intermediate, we subcloned the dominant negative HER-3 fragment into the pCMV bicistronic retroviral expression vector. We first ligated the dominant negative HER-3 fragment into the *Sal*I and *Bam*HI sites located within the extensive polylinker region of pBK-CMV to generate pBK-CMV-dn3. This ligation also introduced an in-frame stop codon 12 codons downstream of the *Bam*HI site. The dominant negative HER-3 fragment was then subcloned from pBK-CMV-dn3 into pCMV by ligation of the dominant negative HER-3 insert cut with *Sal*I and *Cla*I (which does not contain these sites internally) into the *Xho*I and *Cla*I sites (*Sal*I and *Xho*I have compatible ends) within pCMV to generate pCMV-dn3 (Fig. 2*B*). Restriction digest analysis confirmed the proper construction of the vectors.

Culture Infection and Selection of Cell Lines. The H16N-2 and 21 MT-1 cell lines were provided by Dr. Vimla Band through the Dana-Farber Cancer Institute (Boston, MA). For routine culture, the cells were grown in F-12 growth medium containing 10 mM *N*-2-hydroxyethylpiperazine-2-ethane sulfonic acid, antibiotic/antimycotic, 0.5 μg/ml fungizone, 5 mM

ethanolamine, 50 ng/ml sodium selenate, 1 μg/ml hydrocortisone, 5 μg/ml transferrin, 5 μg/ml insulin, 10 ng/ml EGF, 0.1 mg/ml BSA, and 2% fetal bovine serum. The cells were cultured at 37°C with 5% carbon dioxide, and the medium was changed every other day. For subculture, the cells were rinsed in calcium magnesium-free HBSS and then rinsed in 0.05% trypsin plus 0.025% EDTA in calcium magnesium-free HBSS. After aspiration of the trypsin solution, the cells were incubated at 37°C for 5–15 min, and the released cells were immediately resuspended in growth medium for replating in 60- or 100-mm tissue culture plates. For routine culture, the cells were counted with a hemocytometer and plated at a density of 10^4 cells/cm^2. For experiments to test the efficiency of antibiotic resistance (Neo^r) and marker gene (LacZ$^+$) coexpression, monocistronic and bicistronic control vectors were introduced into H16N-2 and 21 MT-1 cells by either transfection or infection. For transfection, the DNA was introduced into cells by lipofection with Lipofectin according to the manufacturer's instructions (Life Technologies, Inc.). For infection, the vectors were first transfected into the ψCRIP packaging cell line followed by infection of target cells with replication-defective virus. Colonies selected for a month on 200 μg/ml G418 (Life Technologies, Inc.) were then fixed and stained for β-galactosidase activity, and the proportion of blue-stained colonies was determined from colony counts. H16N-2 and 21 MT-1 cells were then infected with either the pCMV vector (as a control) or the pCMV-dn3 vector. For these infections, ψCRIP cells were transiently transfected with either pCMV or pCMV-dn3 by lipofection, and medium conditioned for 24 h containing virus was collected and spun down at 1200 rpm for 10 min before adding to subconfluent H16N-2 and 21 MT-1 cell cultures. The H16N-2 and 21 MT-1 cells were then incubated with ψCRIP conditioned medium for 3 days, with fresh conditioned medium added daily. After an additional 2-day incubation in fresh medium, the infected cell lines were then selected on 200 μg/ml G418 for a month before use in further analysis.

Immumocytochemistry. The cells were plated in 24-well plates at a density of 5×10^2 cells/well and cultured to confluence. The cells were rinsed in PBS, fixed in methanol at −20°C for 10 min, and then rinsed three times with PBS before immunostaining with the anti-HER-3 monoclonal antibody, H105 (Neomarkers). The cells were equilibrated in TBS [150 mM NaCl and 50 mM Tris (pH 7.5)], blocked in TBS plus 1% BSA at room temperature for 60 min with mild agitation, and then with 2 μg/ml H105 antibody in TBS plus 1% BSA at room temperature for 60 min with mild agitation. The cells were then rinsed in TBS three times (5 min each time) with moderate agitation, incubated with biotinylated antimouse IgG secondary antibody (Vector Laboratories) at a 1:750 dilution in TBS plus 1% BSA at room temperature for 60 min with mild agitation, rinsed in TBS three times (5 min each time) with moderate agitation, and then incubated with ABC strepavidin HRP reagents (Vector Laboratories) diluted in TBS + 1% BSA at room temperature for 60 min with mild agitation. After rinsing of the cells in TBS three times (5 min each time) with moderate agitation, the cells were stained with DAB as the substrate.

Immunoprecipitations. Cells cultures were incubated in SF medium without insulin and EGF for 48 h before extraction (*i.e.*, the constitutive condition) and stimulation with 10 ng/ml HRG-β for 10 min at 37°C before extraction of lysate protein for immunoprecipitation and/or Western blot analysis. After the cells were lysed in immunoprecipitation buffer [150 mM NaCl, 50 mM Tris (pH 7.5), 0.5% NP40, 5 mM EDTA, 5 mM sodium orthovanadate, 10 mM Na PP_i, and 2 mM phenylmethylsulfonyl fluoride], the lysates were clarified by centrifugation at 14,000 × g for 15 min and either used directly for electrophoresis or used for immunoprecipitation after normalizing the samples. Total cell lysate protein was assayed using the Bradford assay (Bio-Rad), and 2 mg of protein were used for immunoprecipitation, or 100 μg of protein were used directly for electrophoresis. For immunoprecipitation, cell lysates were then incubated with either 30 μl anti-phosphotyrosine monoclonal antibody-conjugated agarose (Oncogene) for 2 h at room temperature with moderate agitation or with 2 μg of 2F12 anti-HER-3 monoclonal antibody (Neomarkers) for 2 h at room temperature with moderate agitation followed by incubation with 50 μl of protein A-agarose (Oncogene) for 1 h at 4°C with moderate agitation. The pellets were then washed three times in immunoprecipitation buffer, and the beads were boiled in 100 μl of electrophoresis sample buffer for 10 min to release protein conjugates from the agarose before electrophoresis.

Western Blot Analysis. Cell lysates or immunoprecipitated samples were electrophoresed in 7.5% SDS-PAGE gels for approximately 18 h at 15 mA constant current. The samples were then transferred to Immobilon-P membranes (Millipore) by overnight electrotransfer in standard transfer buffer at 125 mA followed by 2 h at 325 mA. The blots were equilibrated in TTBS, blocked in TTBS plus 3% milk at room temperature for 60 min with moderate agitation, and then incubated with either 2 μg/ml PY20 anti-phosphotyrosine monoclonal antibody (Oncogene), 1:500 Pab9.3 anti-HER-2 polyclonal antiserum (Berlex Biosciences), 2 μg/ml 2F12 anti-HER-3 monoclonal antibody (Neomarkers), 1:500 anti-p85 polyclonal antiserum (Upstate Biotechnology), or 1:500 anti-SHC polyclonal antiserum (Transduction Laboratories) in TTBS plus 3% milk at room temperature for 60 min with moderate agitation. The blots were then rinsed in TTBS three times (5 min each) with moderate agitation, incubated with biotinylated antimouse IgG or biotinylated antirabbit IgG secondary antibody (Vector Laboratories) at a 1:750 dilution in TTBS at room temperature for 60 min with moderate agitation, rinsed in TTBS three times for 5 min each with moderate agitation, and then incubated with ABC streptavidin HRP reagents (Vector Laboratories) diluted in TTBS at room temperature for 60 min with moderate agitation. After the final rinsing of the blots in TTBS (three times; 5 min each) with moderate agitation, the bands were visualized with enhanced chemiluminescent substrate (Pierce) according to the manufacturer's instructions. Negatives exposed by chemiluminescent substrate were scanned and quantified using the IQ25 Intelligent Quantifier system (Bio Image).

Cell Growth Assays. For the monolayer growth assay, the cell lines were plated in 6-well tissue culture plates at a density of 10^5 cells/well in medium containing all of the supplements listed above minus the insulin and EGF. After 24 h, the medium was replaced with SF medium without growth factors, medium with 5 μg/ml insulin and 10 ng/ml EGF, or medium with 10 ng/ml HRG-β, and the media were changed every other day. Cell counts were taken after 24 h to measure the plating efficiency and at day 10 to measure the proliferation during 9 days in SF culture. For counting cells, the cells from triplicate wells for each condition were trypsinized and counted using a hemocytometer. For the soft agarose assays, the cells were plated in 24-well plates within 0.3% agarose at a density of 2.5×10^4cells/0.25 ml atop a 0.25-ml layer of 0.6% agarose in growth medium with or without 10 ng/ml HRG-β and cultured for a month before counting colonies of at least 50 μm in diameter.

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Heregulin-β Is Especially Potent in Activating Phosphatidylinositol 3-Kinase in Nontransformed Human Mammary Epithelial Cells

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The *neu* differentiation factors/heregulins (HRGs) comprise a family of polypeptide growth factors that activate p185^{erbB-2} through direct binding to either *erb*B-3 or *erb*B-4 receptor tyrosine kinases. We have previously shown that HRG-β is mitogenic for various human mammary epithelial cell lines that coexpress c-*erb*B-2 and c-*erb*B-3. Phosphatidylinositol 3-kinase (PI3K) is activated by p185^{erbB-2}/*erb*B-3 heterodimers in cells stimulated by HRG, and PI3K is constitutively activated by p185^{erbB-2}/*erb*B-3 in breast carcinoma cells that overexpress c-*erb*B-2. To better understand the relative abilities of HRGs, epidermal growth factor (EGF), or insulin to activate PI3K under normal physiological conditions, we compared the levels of recruitment of the 85-kDa regulatory subunit of PI3K when activated by the type I (*erb*B) or type II [insulin-like growth factor (IGF)] receptor tyrosine kinases in two different nontransformed human mammary epithelial cell lines. The nontransformed H16N-2 cells isolated from normal tissue express EGFR, p185^{erbB-2}, and *erb*B-3, and are highly responsive to the mitogenic effects of HRG-β as well as to the combination of EGF and insulin in serum-free culture. We measured the stoichiometry of p85 recruited by tyrosine-phosphorylated proteins induced in H16N-2 cells by either the α or the β isoform of HRG. HRG-β was greater than 10-fold more potent in inducing p85 recruitment than was the less biologically active HRG-α isoform. HRG-β was also a more potent inducer of p85 recruited by tyrosine-phosphorylated proteins than was either EGF, insulin, or EGF and insulin combined. Furthermore, *erb*B-3 principally mediated the direct recruitment of p85 in cells stimulated by HRG or EGF, indicating that, in addition to the high-level activation of PI3K by p185^{erbB-2}/*erb*B-3, EGFR/*erb*B-3 heterodimer interaction is essential for the weak but significant level of PI3K activated by EGF in cells that express normal EGFR levels. Studies using the PI3K inhibitor wortmannin also indicated that PI3K activation was required for the proliferation of H16N-2 cells induced by either HRG-β or EGF and insulin in serum-free culture. Finally, HRG-β was also an especially potent inducer of PI3K in the nontransformed MCF-10A cells, which were derived spontaneously from normal reduction mammoplasty tissue. These data show, for the first time, a side-by-side quantitative comparison of the relative degree of PI3K activated by different growth factors in nontransformed growth factor–dependent cells under precisely defined conditions in culture. J. Cell. Physiol. 183:301–313, 2000.

The PI3K signal transduction pathway is strongly implicated in the growth factor–induced mitogenesis of various cell types through its association with receptor tyrosine kinases. PI3K was first identified as a component of a complex with middle T antigen and pp60$^{c\text{-}src}$ in cells transformed by polyoma virus, and its activity was shown to be required for transformation by middle T antigen (Whitman et al., 1985; Courtneidge and Heber, 1987; Kaplan et al., 1987; Auger et al., 1989). Molecular cloning later showed PI3K to be composed of an 85-kDa regulatory subunit (Escobedo et al., 1991b; Otsu et al., 1991; Skolnik et al., 1991) and a 110-kDa subunit that directly mediates its enzymatic function (Hiles et al., 1992). p85 contains two SH-2 domains that facilitate the binding of PI3K to specific phosphotyrosine residues on both receptor and nonreceptor ty-

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rosine kinases (Escobedo et al., 1991a; Fantl et al., 1992; McGlade et al., 1992). Activation of PI3K involves localization of the enzyme to the plasma membrane, where it phosphorylates PI 4-P and PI 4,5-P in vivo to generate PI 3,4-P_2 and PI-3,4,5-P_3, respectively (Susa et al., 1992). PI3K activation may also be directly associated with serine/threonine kinase activity (Carpenter et al., 1993; Lam et al., 1994) and induces the activation of $p70^{s6k}$ (Chung et al., 1994), as well as certain isoforms of protein kinase C (Nakanishi et al., 1993). PI3K activation is now known to be required for mitogenic responses mediated by many growth factor receptor tyrosine kinases (Varticovski et al., 1994), including platelet-derived growth factor (PDGF) β (Severinsson et al., 1990), colony-stimulating factor (CSF) 1 (Shurtleff et al., 1990), insulin (Cheatham et al., 1994), and EGF (Roche et al., 1994) receptors.

The c-*erb*B-2 (*neu*/HER-2), c-*erb*B-3 (HER-3), and *erb*B-4 (HER-4) genes encode single-subunit transmembrane tyrosine kinases that were originally cloned based on their high homology to the EGF receptor (i.e., EGFR/c-*erb*B-1/HER-1), and together comprise the type I family of receptor tyrosine kinases (Coussens et al., 1985; Kraus et al., 1989; Ullrich and Schlessinger, 1990; Plowman et al., 1993). The *erb*B receptor tyrosine kinases all contain ectodomains with two cysteine-rich sequences. Despite this structural homology, these receptors differ in their ligand specificities. Thus, EGFR binds several distinct EGF-related ligands (e.g., transforming growth factor-α, amphiregulin, and heparin-binding EGF), whereas *erb*B-3 and *erb*B-4 are receptors for more than a dozen different isoforms of the HRGs (Holmes et al., 1992; Wen et al., 1992; Carraway et al., 1994; Tzahar et al., 1994; Schaefer et al., 1998). Although no direct ligand for $p185^{erbB-2}$ has yet been cloned, it is now clear that $p185^{erbB-2}$ is capable of heterodimerization with EGFR (King et al., 1988; Goldman et al., 1990; Wada et al., 1990), *erb*B-3 (Kita et al., 1994; Sliwkowski et al., 1994), and *erb*B-4 (Plowman et al., 1993a,b), and these $p185^{erbB-2}$-containing heterodimers form the highest affinity binding sites for their respective ligands (Wada et al., 1990; Sliwkowski et al., 1994). Differential activation of signal transduction pathways by the various *erb*B receptors is implied by divergence of the carboxyl cytoplasmic domains between the different receptors (Carraway and Cantley, 1994). However, our understanding of how these signals vary according to the participation of various receptor homodimer and heterodimer combinations at differing levels of receptor expression is still fragmentary.

We have previously shown that HRGs are mitogenic for various human mammary epithelial cell lines that coexpress both $p185^{erbB-2}$ and *erb*B-3, but not *erb*B-4 (Ram et al., 1995, 1996). Other studies have shown the relative levels of mitogen-activated protein (MAP)- and c-*jun* (JNK)-kinase activation in response to EGF or HRG in cell lines engineered to express different combinations of the *erb*Bs, or in tumor cell lines known to express high levels of these receptors in culture (Karunagaran et al., 1995, 1996; Pinkas-Kramarski et al., 1996; Tzahar et al., 1996). However, little has been published concerning the quantitative differences in the activation of signal transduction induced by the different *erb*Bs within nontransformed cell populations expressing low but physiologically relevant receptor levels. Nontransformed human mammary epithelial cells require the synergistic action of both IGF-I (or supraphysiological levels of insulin) and EGF to proliferate under serum-free conditions in culture (Stampfer et al., 1980; Hammond et al., 1984; Ethier et al., 1990). Interestingly, our previous studies showed that HRG elicits growth responses in nontransformed human mammary epithelial cells in the absence of both IGF and EGF in serum-free culture (Ram et al., 1995; Ram and Ethier, 1996; Ram et al., 1996). Thus, HRG can mimic the combined mitogenic actions of both IGF and EGF in mammary epithelial cells that coexpress c-*erb*B-2 and c-*erb*B-3. This indicated that the combined actions of $p185^{erbB-2}$ and *erb*B-3 mediate the activation of signal transduction mechanisms that overlap with, or substitute for, those usually requiring the presence of both IGF and EGF. We also found that HRG induces p85 recruitment by both $p185^{erbB-2}$ and *erb*B-3 in nontransformed human mammary epithelial cells, and $p185^{erbB-2}$/*erb*B-3 heterodimers constitutively activate PI3K in breast cancer cell lines with c-*erb*B-2 gene amplification and high level overexpression of $p185^{erbB-2}$ (Ram and Ethier, 1996). Because HRG-β was found to be an especially potent mitogen in the H16N-2 nontransformed human mammary epithelial cell line, further work was also initiated to determine the relative importance of the different type I and type II receptor tyrosine kinases in signal transduction. Here, we have now focused on the quantitative differences in PI3K activation that are elicited by the different isoforms of HRG, EGF, or insulin in these growth factor–dependent cells and other nontransformed human mammary epithelial cells isolated from normal tissue.

MATERIALS AND METHODS

Cell culture

The H16N-2 cell line was provided by Vimla Band through the Dana-Farber Cancer Institute (Boston, MA). The MCF-10A cell line was provided by Dr. Herbert Soule through the Michigan Cancer Foundation (Detroit, MI). The cell lines used in this study were routinely cultured in F-12 medium containing 10 mM *N*-2-hydroxyethylpiperazine-2-ethane sulfonic acid (HEPES), 5 μg/ml gentamycin, 0.5 μg/ml fungizone, 5 mM ethanolamine, 50 ng/ml sodium selenate, 10 mM triiodothyronine, 1 μg/ml hydrocortisone, 5 μg/ml transferrin, 5 μg/ml insulin, 10 ng/ml EGF, 0.1 mg/ml bovine serum albumin (BSA), and 2% fetal bovine serum (FBS). All cells were cultured at 37°C with 10% carbon dioxide in air in a humidified incubator, and the medium was changed every other day. For subculture, the cells were rinsed in calcium magnesium–free Hank's balanced salt solution, then in 0.05% trypsin plus 0.025% EDTA in calcium magnesium–free Hank's balanced salt solution. After aspiration of the trypsin solution, the cells were incubated at 37°C for 5–15 min and the released cells were immediately resuspended in growth medium for replating in 60- or 100-mm tissue culture plates. For routine culture, cells were counted with a Coulter counter and plated at a density of 10^4 cells/cm^2. EGF and insulin were purchased from Sigma (St. Louis, MO) and the α and β isoforms of HRG were provided by Amgen, Inc. (Thousand Oakes, CA).

For inhibition studies, wortmannin (Sigma) was added daily to cultures in DMSO at a 1:1,000 dilution.

Immunoprecipitations

Monolayer cell cultures were incubated in serum-free medium without IGF or EGF for 48 h before induction of cells with growth factors and isolation of cell lysates for immunoprecipitation and Western blot analysis. Cell lysate protein was assayed using the Bradford assay (Bio-Rad, Richmond, CA) and equal amounts of protein were used for immunoprecipitations (2 mg, unless otherwise specified) or directly for electrophoresis (100 μg, unless otherwise specified). Cells lysed in immunoprecipitation buffer [150 mM NaCl, 50 mM Tris (pH 7.5), 0.5% NP-40, 5 mM EDTA, 5 mM sodium orthovanidate, 10 mM sodium pyrophosphate, and 2 mM phenylmethylsulfonyl fluoride] were centrifuged for 15 min at 14,000*g* and either used directly by dilution in electrophoresis sample buffer or for immunoprecipitation after normalizing samples. For immunoprecipitations, cell lysates were incubated with either 30 μl/sample primary antiphosphotyrosine monoclonal antibody-conjugated agarose (Oncogene, Uniondale, NY), or 2 μg/sample anti-*erb*B monoclonal antibodies (Oncogene and Neomarkers, Union City, CA), for 2 h at room temperature with agitation. For the unconjugated antibodies, primary antibody binding was followed by incubation with 50 μl protein A- or G-agarose solution (Sigma) for 1 h at 4°C before washing three times in immunoprecipitation buffer and boiling the beads in sample buffer for 10 min to release protein conjugates from the agarose.

Western blot analysis

Cell lysates or immunoprecipitated samples were electrophoresed in 7.5% SDS–PAGE gels for approximately 20 h at 15 mA constant current. The samples were then transferred to Imobilon-P membranes (Millipore, Bedford, MA) by overnight electrotransfer in standard transfer buffer at 125 mA followed by 2 h at 325 mA. The blots were equilibrated in Tween/Tris-buffered saline [TTBS; 150 mM NaCl, 50 mM Tris (pH 7.5) plus 0.1% Tween-20], incubated in TTBS containing 3% nonfat dry milk at room temperature for 60 min with mild agitation and incubated with anti-p85 (Upstate Biotechnology, Lake Placid, NY) or anti-*erb*B antibodies (Oncogene, Neomarkers, and Berlex Biosciences, Richmond, CA) at a 1:500 dilution in TTBS plus 3% milk at room temperature for 60 min. The blots were then rinsed in TTBS three times with moderate agitation for 5 min after each antibody binding step. Biotinylated anti-rabbit or anti-mouse IgG secondary antibody was used at a 1:1,000 dilution and "ABC" strept-avidin horseradish peroxidase reagents (Vector Laboratories, Burlingame, CA) were used to visualize bands with enhanced chemiluminescence (Pierce, Rockford, IL), according to the manufacturer's instructions.

Flow cytometry

The cells were rinsed once in F-12 plus 0.1% BSA (F-12 + BSA) before labeling with 1 μg/ml primary monoclonal antibody directed against either IgG, EGFR, p185$^{erbB\text{-}2}$, or *erb*B-3 in F-12 + BSA for 60 min at 4°C with mild agitation. Cells were rinsed twice with F-12 and then incubated with 2 μg/ml biotinylated anti-mouse secondary antibody in F-12 + BSA for 60 min at 4°C with mild agitation. Cells were rinsed twice with PBS (pH 7.4) and then incubated with FITC-conjugated streptavidin in PBS according to the manufacturer's instructions (Vector Laboratories) for 30 min at 4°C with mild agitation in the dark. The labeled cells were then rinsed twice with PBS and detached from the culture plates by a very brief incubation with trypsin/EDTA solution (this and all remaining steps were performed under low-light conditions). The detached cells were then transferred to 15-ml centrifuge tubes and spun at 1,500 rpm for 10 min at 4°C. The supernatant was removed and the cell pellets were then resuspended with gentle trituration and transferred to a small glass tube in PBS. The cells were run through standard FACS analysis and separated according to the initial determination of a 100-μl test sample.

PI3K enzymatic assay

Assay for PI3K enzymatic activity employed radioisotope incorporation into phosphotidylinositol (Sigma) and thin-layer chromatography (TLC) as previously described (Hu et al., 1992).

Proliferation assay

For the growth experiments, H16N-2 cells were plated in six-well tissue culture plates at a density of 10^5 cells/well (i.e., 10^4 cells/cm^2) in medium containing all of the factors listed earlier minus the insulin and EGF. After 24 h, the medium was replaced with serum-free medium containing the other supplements with either 10 ng/ml HRG-β, or 10 ng/ml EGF and 5 μg/ml insulin to stimulate growth. At this time, DMSO at a 1:1,000 dilution was either added alone (as a control) or containing wortmannin at various concentrations. The cells were then fed daily with fresh growth factor(s) and wortmannin in DMSO. Cell counts were taken after 24 h to measure the plating efficiency and at day 10 to measure the proliferation during 9 days in culture from the time of growth factor and wortmannin addition. For counting cells, the cell nuclei from triplicate wells for each condition were detergent-solubilized and counted in a Coulter counter as previously described (Ethier et al., 1990).

RESULTS

Expression and function of *erb*B receptor tyrosine kinases in the H16N-2 nontransformed human mammary epithelial cell line

The H16N-2 human mammary epithelial cell line was derived from normal tissue by introduction of the human papilloma virus (HPV) into normal primary mammary epithelial cells in early passage culture (Band et al., 1990; Band and Sager, 1991). We previously used this cell line as a control to study the growth factor requirements and activation of signal transduction pathways in a series of breast carcinoma cell lines originally isolated from the same patient (Ram and Ethier, 1996; Ram et al., 1996). In the course of these earlier studies, we found that exogenous HRG-β induced especially potent mitogenic responses in these cells (Ram and Ethier,

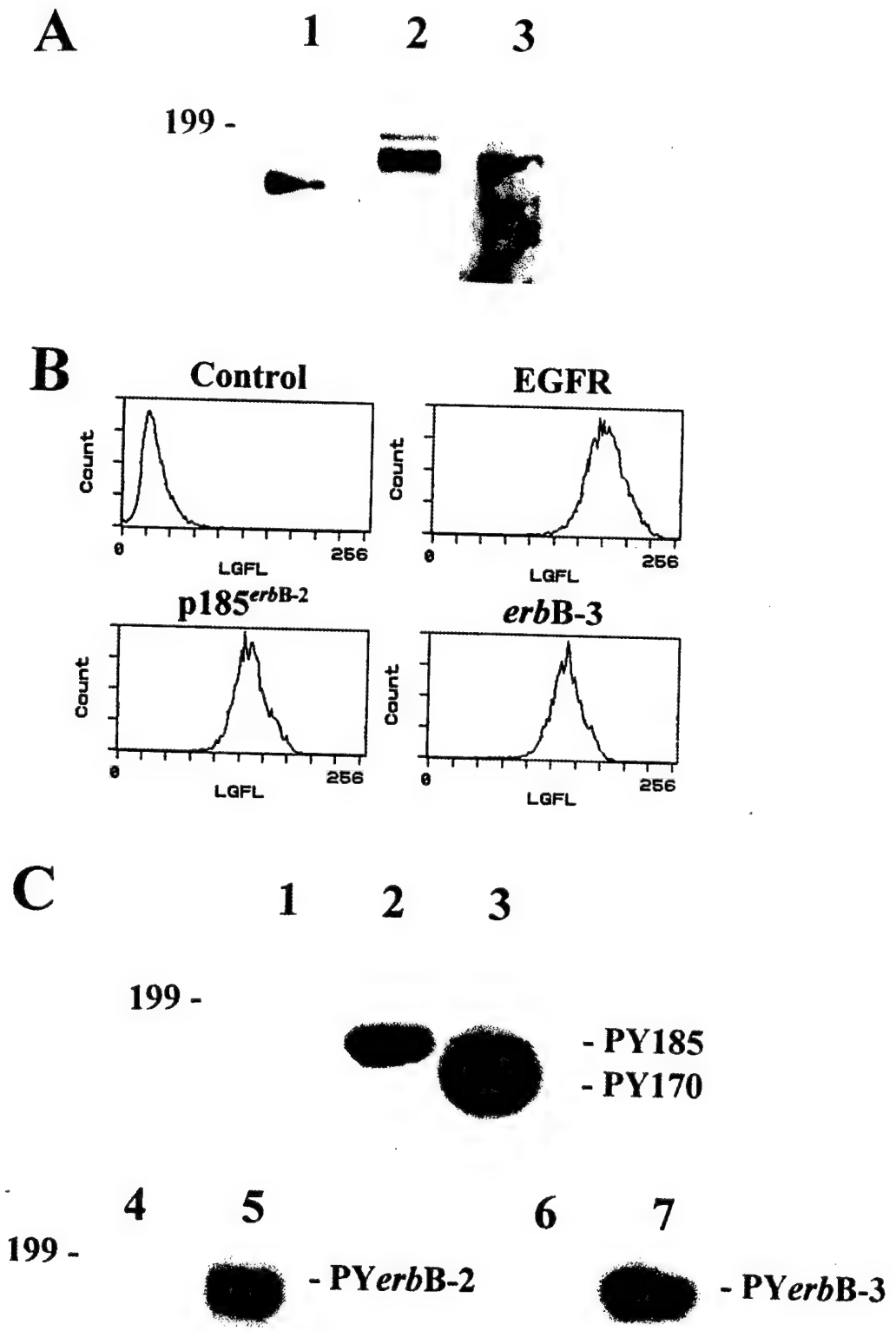

Fig. 1. EGFR, p185^{erbB-2}, and *erb*B-3 expression and activation in H16N-2 cells. **A:** Immunoprecipitation/Western blot analysis of EGFR (lane 1), p185^{erbB-2} (lane 2), and *erb*B-3 (lane 3) expression by H16N-2 cells in culture. Immunoprecipitation was performed using cell lysates containing 2 mg protein with anti-EGFR, anti-p185^{erbB-2}, and anti-*erb*B-3 monoclonal antibodies followed by Western blotting for each receptor separately. **B:** Flow cytometry measurement of cell surface EGFR, p185^{erbB-2}, and *erb*B-3 on H16N-2 cells in culture. **C:** Antiphosphotyrosine Western blot analysis of receptor phosphorylation in H16N-2 cells in serum-free medium without growth factors for 48 h (lane 1), and after 10 min stimulation with 10 ng/ml of the 5-kDa forms of HRG-β (lane 2) or EGF (lane 3). Antiphosphotyrosine immunoprecipitation was also followed by anti-p185^{erbB-2} Western blot analysis (lanes 4 and 5), and anti-*erb*B-3 immunoprecipitation was followed by antiphosphotyrosine Western blot analysis (lanes 6 and 7), showing the level of p185^{erbB-2} and *erb*B-3 phosphorylated in H16N-2 cells without (lanes 4 and 6) and with (lanes 5 and 7) HRG stimulation.

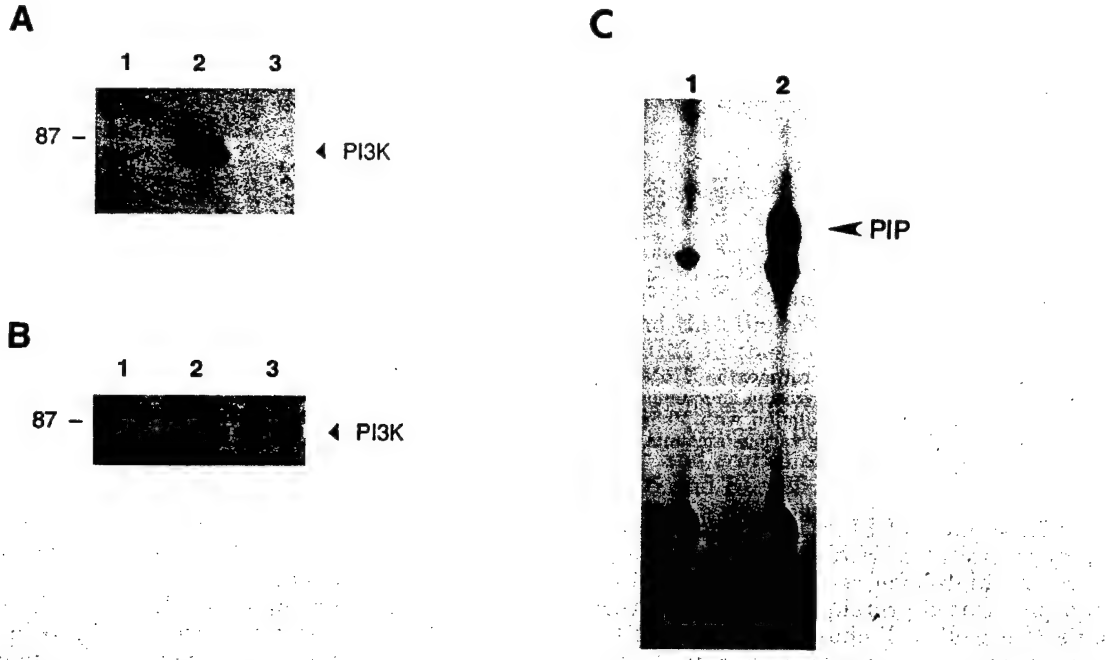

Fig. 2. PI3K activation in response to HRG in H16N-2 cells in culture. **A:** Antiphosphotyrosine immunoprecipitation was followed by Western blotting with anti-p85 antiserum to measure the recruitment of $p85^{PI3K}$ by tyrosine-phosphorylated proteins in H16N-2 in serum-free medium without growth factors for 48 h (lane 1), in cells then stimulated with HRG-β for 10 min in culture (lane 2), and for cell lysate to which HRG-β was added subsequent to cell lysate preparation (lane 3). **B:** Western blot of aliquots taken from the same sample before immunoprecipitation were directly blotted with anti-p85 antiserum as a control to ensure equal loading of the samples. **C:** PI3K enzymatic assay in which antiphosphotyrosine immunoprecipitation of 2 mg cell lysate protein was followed by incubation in a kinase reaction with ^{32}P-ATP and phosphatidylinositol (PI), followed by visualization with TLC to identify the PI 3′-phosphorylated (PIP) product for H16N-2 cells in serum-free medium without any growth factors for 48 h (lane 1), and for cells then stimulated with HRG-β (lane 2) for 10 min in culture.

1996). Although there are not any previous reports showing a direct comparison of PI3K induction by EGF- and IGF-related growth factors under comparable well-defined conditions, our earlier studies did suggest that the H16N-2 cell line offers an ideal model system to do such studies in cells derived from normal tissue. Northern blotting and reverse-transcriptase polymerase chain reaction (RT-PCR) characterization of mRNA expression have shown that these cells express c-*erb*B-1, c-*erb*B-2, and c-*erb*B-3, but not c-*erb*B-4 (Band et al., 1990; Ram et al., 1996). Here, we employed immunoprecipitation of large quantities of lysate protein in conjunction with Western blotting to identify the presence of EGFR, $p185^{erbB-2}$, and *erb*B-3 in the H16N-2 cells (Fig. 1A). H16N-2 cells expressed very low but detectable levels of these receptors when compared to other lines known to overexpress these receptors. Also, detectable levels of cell surface EGFR, $p185^{erbB-2}$, and *erb*B-3 were measured in the H16N-2 cells using flow cytometry (Fig. 1B). Antiphosphotyrosine Western blots showed high levels of $p185^{erbB-2}$/*erb*B-3 tyrosine phosphorylation induced by HRG (Fig. 1C, lane 2) and EGFR tyrosine phosphorylation induced by EGF (Fig. 1C, lane 3) in H16N-2 cells in culture. We also confirmed the tyrosine phosphorylation of both $p185^{erbB-2}$ (Fig. 1C, lane 5) and *erb*B-3 (Fig. 1C, lane 7) induced by HRG in H16N-2 cells using immunoprecipitation followed by Western blotting to measure receptor phosphorylation separately.

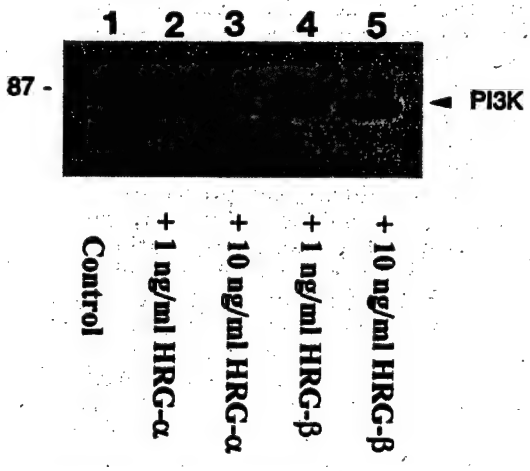

Fig. 3. Comparison of p85 recruitment induced by α or β HRG isoforms in H16N-2 cells. Antiphosphotyrosine immunoprecipitation was followed by Western blotting with anti-p85 antiserum to measure the relative level of p85 recruitment in cells without growth factors (lane 1), or for cells stimulated with 1 ng/ml HRG-α1 (lane 2), 10 ng/ml HRG-α1 (lane 3), 1 ng/ml HRG-β1 (lane 4), or 10 ng/ml HRG-β1 (lane 5) for 10 min in culture.

Activation of PI3K by HRGs in H16N-2 cells in culture

We studied the activation of the PI3K pathway in H16N-2 cells using both a PI3K enzymatic assay as well as by directly measuring the recruitment of the

Fig. 4. Stoichiometry of p85 recruitment in H16N-2 cells stimulated with HRG-β. **A:** Antiphosphotyrosine immunoprecipitations from H16N-2 cell lysates after stimulation with 10 ng/ml HRG-β were titrated by immunoprecipitating various amounts of cell lysate protein followed by Western blotting with anti-p85 antiserum. **B:** Various amounts of lysate protein were also loaded straight into the gel before immunoprecipitation to quantify the relative level of p85 recruitment in cells stimulated with HRG-β.

85-kDa subunit of PI3K in antiphosphotyrosine immunoprecipitates. HRG-β was found to potently induce the high-level recruitment of p85 by tyrosine-phosphorylated proteins in cells in culture (Fig. 2A, lane 2) as well as 3′ phosphorylation of PI products in antiphosphotyrosine immunoprecipitates (Fig. 2B, lane 2). We have found anti-p85 blotting of antiphosphotyrosine immunoprecipitates to provide the most reliable and quantitative measure of PI3K activation that doesn't involve detectable tyrosine phosphorylation of p85 itself, but rather recruitment of PI3K by activated receptor complexes (Ram and Ethier, 1996). We also investigated the possibility that recruitment of p85 by antiphosphotyrosine immunoprecipitation may actually occur in vitro during the immunoprecipitation procedure. However, no detectable recruitment of p85 was seen in antiphosphotyrosine immunoprecipitates when HRG was added after the cells were lysed (Fig. 2A, lane 3). Therefore, we conclude that the induction of p85 recruitment by tyrosine-phosphorylated proteins was occurring exclusively in vivo during the procedure, because this association could not be induced by growth factor added subsequent to lysate preparation.

We assessed the relative potency of the different isoforms of HRGs for inducing PI3K recruitment by activated *erb*B receptor tyrosine kinase complexes. Direct comparisons of p85 recruitment in antiphosphotyrosine immunoprecipitates were measured for H16N-2 cells stimulated by either the α or the β isoform of HRG at various concentrations. As shown in Fig. 3, the HRG-β1 isoform induced levels of p85 recruitment with more than 10-fold greater potency than did HRG-α1. Also, Western blots of aliquots taken from the same samples before immunoprecipitation were directly blotted with anti-p85 antiserum as a control to ensure equal loading of samples in this and other such experiments described later (data not shown). The high-level induction of p85 in response to HRG-β was associated with a much greater mitogenic response of H16N-2 cells to HRG-β in culture (data not shown), as we have previously reported for the MCF-10A human mammary epithelial cell line in culture (Ram et al., 1995). To investigate the stoichiometry of p85 recruited by phosphotyrosine-containing complexes during stimulation with HRG, we titrated various amounts of lysates for immunoprecipitation (Fig. 4A) in conjunction with anti-p85 blotting of Western blots containing nonimmunoprecipitated lysates (Fig. 4B) taken directly from the same sample. As shown in Fig. 4, approximately 2–4% of the total amount of p85 in the cell was recruited by antiphosphotyrosine immunoprecipitates during activation in response to HRG-β (i.e., more than 20-fold the amount of lysate protein must be immunoprecipitated to see a signal comparable to that seen in simple Western blots in side-by-side comparisons). In addition, we confirmed the quantitative nature of the assay by showing that reimmunoprecipitation of supernatants (Fig. 4A, lane 4) after initial immunoprecipitation with antiphosphotyrosine antibody did not show any detectable p85, indicating that the antibody used in these experiments was not saturated under these immunoprecipitation conditions using up to 2 mg of lysate protein.

Comparison of PI3K activation induced by HRG-β, EGF, or insulin in H16N-2 cells

Whereas our previous work suggested that HRG may be an especially potent activator of PI3K compared to other growth factors, this proposal had not yet been tested directly. Therefore, we measured the level of PI3K induced by the various receptor tyrosine kinases in H16N-2 cells stimulated by different growth factors. The measurement of p85 recruitment by antiphosphotyrosine immunoprecipitation is a technique well suited to compare levels of PI3K activation by the different receptor tyrosine kinases, because this technique efficiently detects signaling molecules associated with intermediary substrates (such as the IRS-1 protein involved in IGF and insulin receptor responses) (Backer et al., 1992; Yamamoto et al., 1992), as well as those recruited directly by the receptor kinases themselves. Therefore, direct side-by-side quantification of p85 recruited by activated complexes during stimulation with EGF- and/or IGF-related growth factors can be directly compared by this method. After 10-min stimulation with different growth factors, HRG-β was found to be especially potent in its ability to induce the recruitment of p85 in antiphosphotyrosine immunoprecipitates (Fig. 5A, lane 2) compared with that for EGF (Fig. 5A, lane 3) or insulin (Fig. 5A, lane 4).

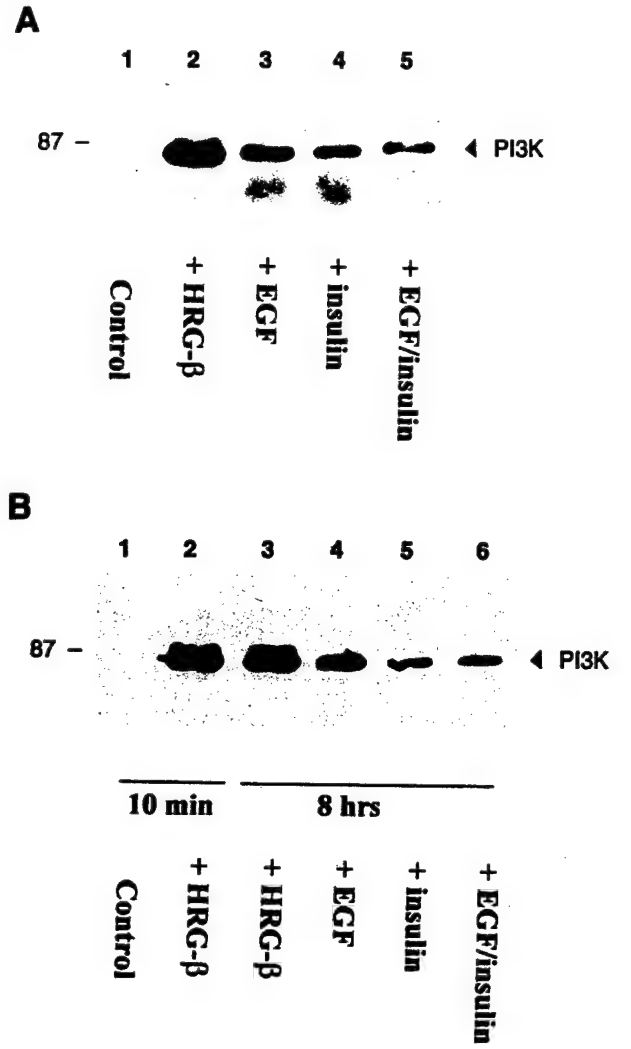

Fig. 5. Comparison of p85 recruitment induced by HRG-β, EGF, and insulin in H16N-2 cells. **A:** Antiphosphotyrosine immunoprecipitations from H16N-2 cell lysates were followed by Western blotting with anti-p85 antiserum to measure the relative level of p85 recruitment in cells without growth factors (lane 1), or for cells stimulated with 10 ng/ml HRG-β (lane 2), 10 ng/ml EGF (lane 3), 5 μg/ml insulin (lane 4), or EGF and insulin combined (lane 5) for 10 min in culture. **B:** The same experiment was performed comparing cells without growth factors (lane 1) or stimulated with HRG-β (lane 2) for 10 min, with that for cells stimulated for 8 h with HRG-β (lane 3), EGF (lane 4), insulin (lane 5), or EGF and insulin combined (lane 6).

Although insulin (at a concentration that maximally activates both IGF-1 and insulin receptors) also induced p85 recruitment, the level of induction with insulin was always significantly less than that seen with either HRG-β or EGF in H16N-2 cells (Fig. 5A). In addition, HRG-β induced a much higher level of p85 recruitment than that seen even when cells were stimulated simultaneously with both EGF and insulin (Fig. 5A, lane 5). We also measured the level of p85 recruitment after incubation with the different growth factor combinations for up to 8 h (Fig. 5B, lanes 3–6), and the same relative level of PI3K induction by the different growth factors was seen.

Differential p85 recruitment by EGFR, p185^{erbB-2}, and *erb*B-3 during ligand-induced activation of PI3K in H16N-2 cells

To directly compare the relative level of p85 recruitment by EGFR, p185^{erbB-2}, and *erb*B-3 when activated by either EGF or HRG-β, we immunoprecipitated the different *erb*B receptor kinases followed by anti-p85 blotting (Fig. 6). This allowed us to measure the levels of p85 directly associated with the different *erb*Bs during activation with either HRG-β or EGF at equimolar concentrations (5-kDa forms of each were used). We have also found that the heterodimers induced by either EGF or HRG in these cells are not stable under these immunoprecipitation conditions, while high-affinity signaling molecule recruitment is maintained (data not shown). Whereas p85 directly associated with both p185^{erbB-2} and *erb*B-3 after activation with HRG (Fig. 6, lanes 5 and 8), p85 was weakly recruited by EGFR and *erb*B-3 in response to stimulation with EGF (Fig. 6, lanes 3 and 9). Only very small increases in the association of p85 with EGFR were seen in cells stimulated by HRG (Fig. 6, lane 2), or with p185^{erbB-2} in cells stimulated by EGF (Fig. 6, lane 6). Although p85 associated mostly with *erb*B-3 in cells stimulated by either HRG or EGF, p185^{erbB-2} also directly associated with p85 to a moderate extent in response to HRG. The direct association of p85 with EGFR during stimulation with EGF (Fig. 6, lane 3) was low but reproducible. Thus, the order of decreasing ability to recruit p85 by the different *erb*B receptor tyrosine kinases was *erb*B-3 > p185^{erbB-2} > EGFR.

Wortmannin inhibition of HRG and EGF/insulin-induced cell proliferation in H16N-2 cells in culture

To test for the requirement of PI3K activation in the proliferative responses seen in these studies, we investigated the effects of a PI3K inhibitor on the proliferation of H16N-2 cells grown either in the presence of HRG-β or with the combination of EGF and insulin. Wortmannin is known to be a specific inhibitor of PI3K at submicromolar concentrations, and was found to inhibit the proliferation of H16N-2 cells in a dose-dependent manner at submicromolar concentrations for cells cultured either with HRG-β or with EGF and insulin (Fig. 7). It was necessary to add the wortmannin in DMSO daily to get effective inhibition (data not shown), which likely results from the known lability of wortmannin once added to aqueous solutions. Also, the inhibitory effects of wortmannin on cell proliferation were reversible, in that cells exposed to wortmannin grew well after washing away the drug (data not shown), indicating that the inhibition of cell proliferation was the result of specific effects, rather than to any more general toxicity of the drug.

Comparison of PI3K activation induced by HRG-β, EGF, or insulin in MCF-10A cells

We also questioned whether the relative levels of p85 recruitment that were seen in the H16N-2 cells were reflective of the general case in normal mammary cells or were a unique feature of this particular cell line. Therefore, we performed similar experiments using the nontransformed human mammary epithelial cell line

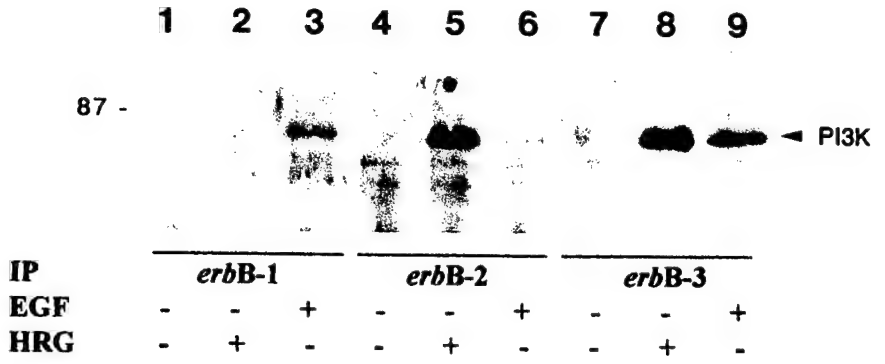

Fig. 6. Comparison of p85 recruitment by EGFR, $p185^{erbB-2}$, and *erb*B-3 induced by EGF or HRG in H16N-2 cells. Anti-EGFR (lanes 1–3), anti-*erb*B-2 (lanes 4–6), and anti-*erb*B-3 (lanes 7–9) immunoprecipitations from H16N-2 cell lysates were followed by Western blotting with anti-p85 antiserum to measure the relative level of p85 recruitment by the different *erb*B receptor tyrosine kinases in cells without growth factors (lanes 1, 4, and 7), in cells stimulated with HRG-β (lanes 2, 5, and 8), or in cells stimulated with EGF (lanes 3, 6, and 9) for 10 min in culture.

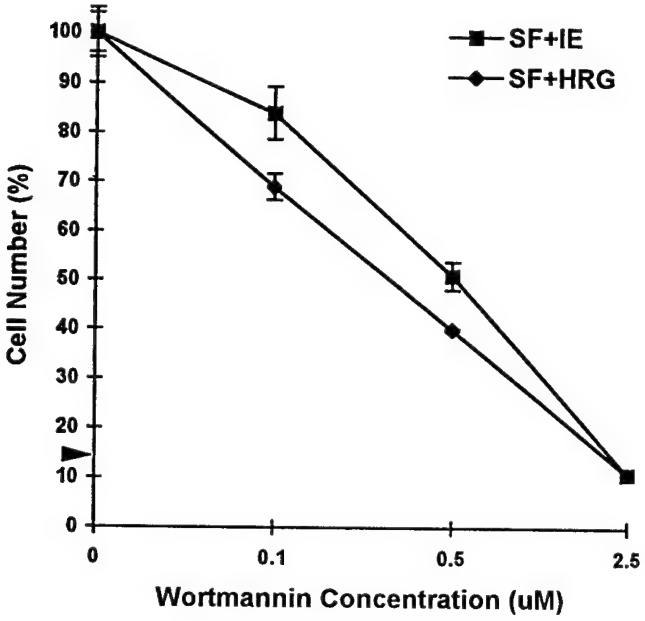

Fig. 7. Wortmannin inhibition of HRG-induced and EGF/insulin-induced cell growth in culture. Growth experiment measuring the proliferation of H16N-2 cells with and without wortmannin by counting cells cultured for 9 days in serum-free medium containing either HRG-β, or EGF and insulin, as well as DMSO alone or containing various concentrations of wortmannin. The average cell number plated after 24 h is indicated by an arrow on the *y* axis. The mean averages of triplicate determinations and SD are shown for each condition.

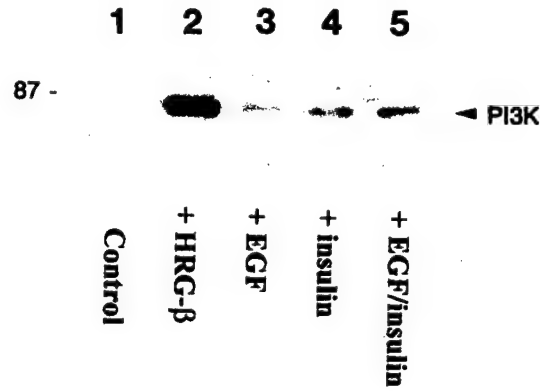

Fig. 8. Comparison of p85 recruitment induced by HRG-β, EGF, and insulin in MCF-10A cells. Antiphosphotyrosine immunoprecipitations from MCF-10A cell lysates were followed by Western blotting with anti-p85 antiserum to measure the relative level of p85 recruitment in cells without growth factors (lane 1), or for cells stimulated with HRG-β (lane 2), EGF (lane 3), insulin (lane 4), or EGF and insulin combined (lane 5) for 10 min in culture.

MCF-10A, which was derived spontaneously from normal tissue without the introduction of HPV (Soule et al., 1990), and is known to have wild-type p53 and retinoblastoma gene function (Merlo et al., 1995; unpublished data). MCF-10A cells also express EGFR, $p185^{erbB-2}$, and *erb*B-3, but not *erb*B-4, and show mitogenic responsiveness to HRG and EGF or insulin in culture (Ram et al., 1995). As shown in Fig. 8, HRG-β was also especially potent in inducing p85 recruitment by antiphosphotyrosine immunoprecipitates in the MCF-10A cells in culture. Therefore, the relative level of PI3K activation in H16N-2 cells is similar to that seen in other cells also isolated from normal mammary tissue that were not immortalized with HPV. Scanning densitometry analysis of p85 recruitment in antiphosphotyrosine immunoprecipitates showed the relative levels of PI3K activation in H16N-2 and MCF-10A cells after stimulation with the different growth factors (Fig. 9). These combined data support the contention that HRG-β is consistently the most effective inducer of PI3K recruitment relative to that seen after stimulation with EGF and/or insulin in various nontransformed human mammary epithelial cell lines.

DISCUSSION

Although much is now known about the ligand specificities and kinase potential of the different type I receptor tyrosine kinases, the functional significance of receptor heterodimer interactions in normal cell growth and development has not yet been as well studied, and little has been done to measure receptor activation and signaling in cells that express physiological receptor levels. For the studies reported here, we used the nontransformed H16N-2 and MCF-10A human

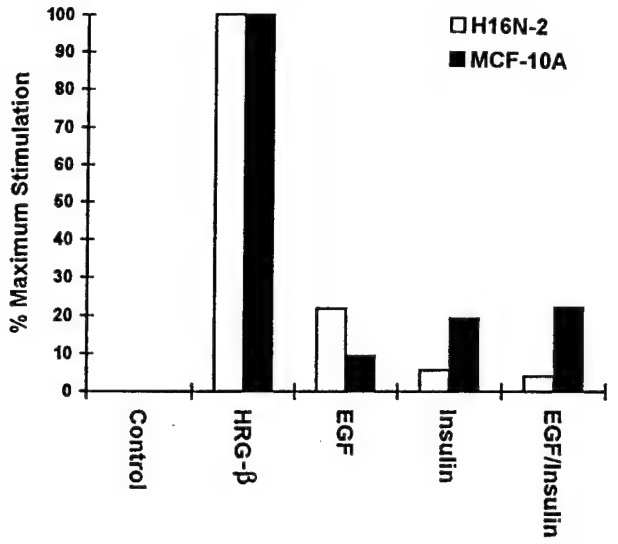

Fig. 9. Comparison of the relative levels of PI3K activation by different growth factors in H16N-2 and MCF-10A cells. Scanning densitometry determination of $p85^{PI3K}$ recruitment in antiphosphotyrosine immunoprecipitates collected from H16N-2 and MCF-10A cells stimulated with HRG-β, EGF, insulin, or EGF and insulin combined. The control comprises cells that were incubated for 48 h in the absence of any growth factors in serum-free medium. The cells were then stimulated for 10 min with either HRG-β, EGF, insulin, or EGF and insulin combined. The levels are expressed as the percentage of p85 recruitment measured relative to the maximal stimulation seen with HRG-β.

mammary epithelial cell lines originally isolated from normal tissues. By using these cell lines, it is possible to study the activation of signal transduction pathways under completely defined serum-free conditions in culture (Ram et al., 1995; Ram and Ethier, 1996; Ram et al., 1996). In the present study, we found that HRG-β was an especially potent activator of PI3K at physiological concentrations in nontransformed human mammary epithelial cells compared to either HRG-α, EGF, or insulin.

Whereas previous studies have shown that the PDGF-β receptor potently activates various signal transduction pathways, including MAP-kinase, phospholipase C (PLC)-γ, and PI3K in genetically engineered cell lines (Escobedo et al., 1991), EGF was shown to only weakly activate PI3K under comparable conditions (Hu et al., 1992). Also, insulin activation of the insulin receptor or IGF-1 receptor was shown to stimulate p85 recruitment through the insulin receptor/IRS-1 complex (Lam et al., 1994). Previous reports have studied responsiveness to these growth factors separately in artificially constructed lines that express very high levels of the receptors (to allow for easy detection) and were done under less well defined serum-containing culture conditions, often also using supraphysiological ligand concentrations. The responses measured in genetically engineered cell lines under such conditions, although advantageous for studying receptor activation under perturbative conditions, may represent conditions more typically associated with pathological states rather than normal responses involving low-level receptor expression. Our present data now demonstrate that HRG-β induction of $p185^{erbB-2}$/*erb*B-3 potently activates PI3K, whereas EGF or insulin only weakly activate PI3K in H16N-2 and MCF-10A cells in side-by-side comparisons. This indicates that the potent activation of PI3K by HRG-β is a common response in nontransformed cells expressing physiological receptor levels, and that for mammary epithelial cells (known to rarely express PDGF receptors), HRG-β is the most potent activator of PI3K yet identified.

We also measured the direct recruitment of PI3K by the different *erb*Bs in response to HRG or EGF by separately immunoprecipitating EGFR, $p185^{erbB-2}$, and *erb*B-3. We found that all three were able to recruit p85 to some extent. However, *erb*B-3 appears to be the predominant receptor able to activate PI3K in response to either HRG or EGF (Fig. 6). Sequence analysis has shown that the cytoplasmic region of *erb*B-3 contains seven YXXM sequences that, when tyrosine-phosphorylated, are known to form the high-affinity binding sites for the SH-2 regions of p85 (Kraus, 1989; Fantl et al., 1992). Previous studies have also confirmed that the cytoplasmic domain of *erb*B-3 is especially potent in activating PI3K when EGFR-*erb*B-3 chimeric receptors are activated by EGF (Fedi et al., 1994). Our more recent analysis has extended these observations and shown that both $p185^{erbB-2}$ and *erb*B-3 contribute to the HRG-induced activation of PI3K, as well as the constitutive activation of PI3K seen in breast cancer cells with c-*erb*B-2 gene amplification (Ram and Ethier, 1996). Furthermore, sequence analysis had shown previously that all four *erb*B kinases contain at least one YXXM sequence (Plowman et al., 1993a) and $p60^{c-src}$ is now known to phosphorylate multiple sites on EGFR and $p185^{erbB-2}$ (including the one YXXM sequence found in these receptors) that are not efficiently phosphorylated through an autophosphorylation mechanism (Stover et al., 1995). Therefore, the lower-level recruitment of PI3K directly by EGFR and $p185^{erbB-2}$ may require the activation of $p60^{c-src}$ as well.

*erb*B-3 is transactivated by EGFR in A431 and MDA-MB-468 breast carcinoma cells stimulated by EGF (Kim et al., 1994; Soltoff et al., 1994). These studies raised the important point that EGFR/*erb*B-3 heterodimers may be required for weakly efficient activation of PI3K by EGF. However, there still remained some doubt about the general relevance of such a mechanism in normal development, because both A431 and MDA-MB-468 cells have EGFR gene amplification and highly overexpress EGFR. In other studies of *erb*B interactions, the cells that were employed were genetically engineered and highly overexpressed the receptors to allow for easy detection, and PI3K activation was not directly measured (Karunagaran et al., 1995, 1996; Pinkas-Kramarski et al., 1996; Tzahar et al., 1996). However, our observations now confirm that the EGFR/*erb*B-3 interaction is operative in cells in which the receptors are not highly overexpressed, and show that *erb*B-3 is almost entirely responsible for the activation of PI3K in cells stimulated by EGF. Furthermore, we have now directly compared this activation with that seen through $p185^{erbB-2}$/*erb*B-3 heterodimers induced in the same cells by HRG-β. From these studies, a hierarchy of PI3K association with the different *erb*Bs in nontransformed mammary epithelial cells is seen: *erb*B-3 > $p185^{erbB2}$ > EGFR in their respective ability to directly associate with p85 during activation.

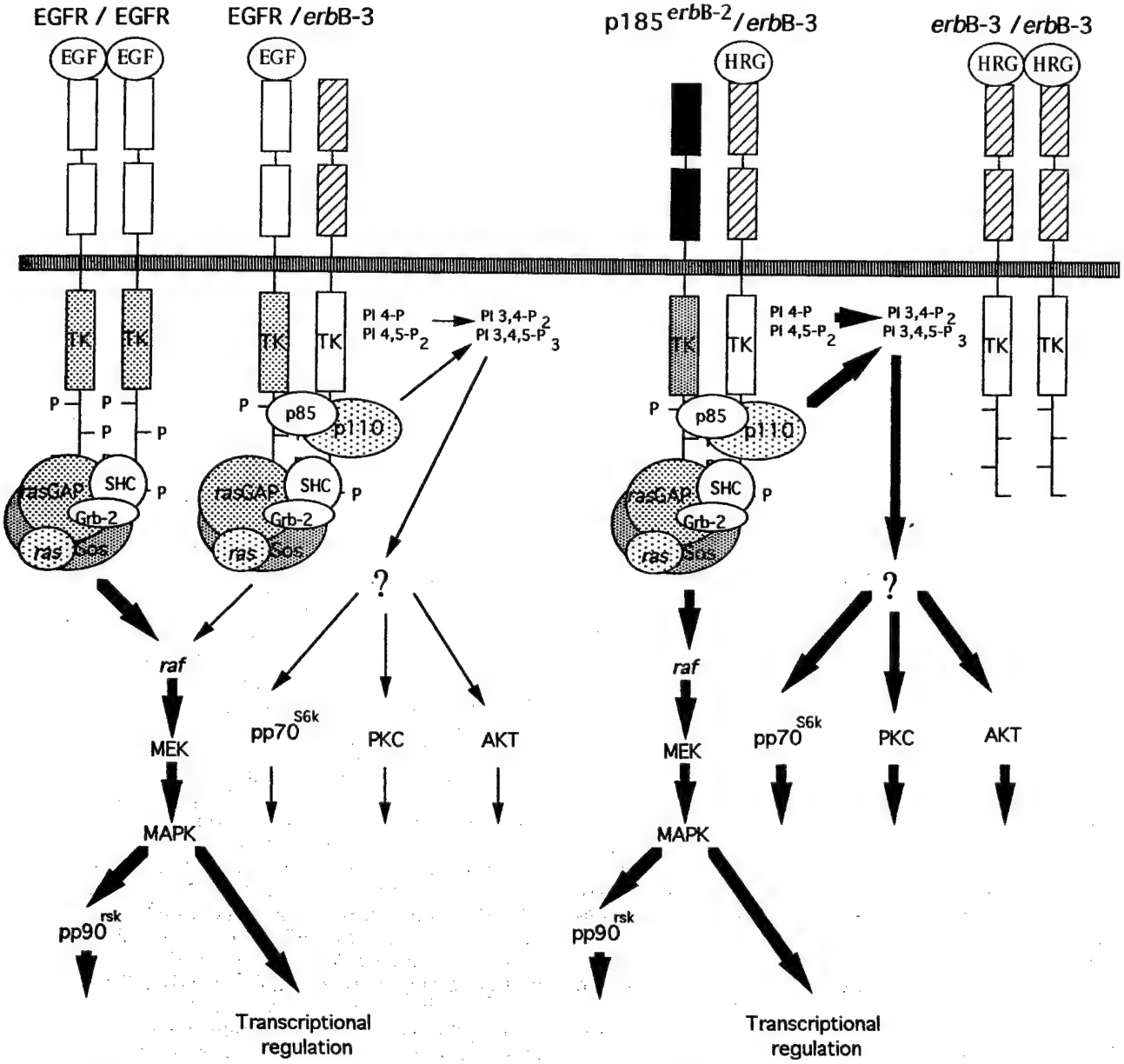

Fig. 10. Model of signal transduction induced by EGF or HRG in nontransformed human mammary epithelial cells. This diagram is based on our own data and a number of references cited throughout the text showing the *erb*B interactions that lead to the induction of PI3K and MAP-kinase activation in cells that express EGFR, $p185^{erbB-2}$, and *erb*B-3 (but not *erb*B-4). The thickness of the arrows indicates the relative strengths of signal activation. Notice that HRG is especially potent in activating both PI3K and MAP-kinase by $p185^{erbB-2}$/*erb*B-3, but *erb*B-3/*erb*B-3 homodimers are kinase deficient. In contrast, EGF activates high levels of MAP-kinase by EGFR/EGFR, but only weakly activates PI3K through EGFR/*erb*B-3 heterodimers. Additionally, although not included in the diagram, a small amount of additional MAP-kinase is likely activated by EGFR/*erb*B-2 stimulated by EGF, as well as PI3K and MAP-kinase activated by EGFR/*erb*B-3 stimulated by HRG; however, these would be only minor contributions to the total integrated signal because the dimer affinities of these species are relatively low compared to the $p185^{erbB-2}$/*erb*B-3 heterodimer and the homodimer interactions. Whereas the dimer affinity of EGFR/*erb*B-3 is also known to be low (i.e., the relative number of such species would be much lower than for the other three that are shown), EGFR/*erb*B-3 still facilitates the weaker but significant activation of PI3K induced by EGF.

Whereas HRG shows the ability to stimulate higher levels of PI 3-kinase recruitment, our experiments with wortmannin suggest that even lower levels of PI 3-kinase activation induced by EGF and insulin are apparently still required for mitogenesis. However, when considering mitogenesis in normal growth factor–dependent cells, the activation of multiple signaling pathways is known to be required for mitogenesis, and the quantitative aspect of activation of various signaling pathways should be considered. In this study we have focused on PI 3-kinase, but we do not contend that PI 3-kinase is alone sufficient to trigger mitogenesis in normal mammary epithelial cells. For example, both EGF and HRG-β are known to strongly activate the MAP-kinase pathway as well

(Marte et al., 1995; Karunagaran et al., 1996; Pinkas-Kramarski et al., 1996). Furthermore, this is consistent with our own data using H16N-2 cells, in which both EGF and HRG were able to induce comparably high-level SHC protein recruitment in antiphosphotyrosine immunoprecipitates (data not shown). The redundancy of mitogenic signaling should also be considered here, in which the integrated signal is determined by various signaling pathways whose quantitative differences may exhibit a substitution phenomenon to achieve threshold levels required for cell growth. In addition, it is known that for cells that show a synergistic requirement for EGF and insulin, EGF is known to be required early in G1, whereas insulin is required late in G1 (Aaronson, 1991), indicating that the temporal sequence of action must also be considered for understanding mitogenesis.

Studies employing genetically engineered cell lines and cross-linking analysis previously showed that the higher-affinity p185$^{erbB\text{-}2}$/*erb*B-3 heterodimer interaction is especially potent in activating the MAP- and JNK-kinase pathways (Karunagaran et al., 1996; Pinkas-Kramarski et al., 1996; Tzahar et al., 1996). However, their results did not measure PI3K activation. Our combined results now support a model in which both the PI3K and MAP-kinase pathways are potently induced by p185$^{erbB\text{-}2}$/*erb*B-3 heterodimers (Fig. 10). Interestingly, the parameters of receptor function, such as homo/heterodimer affinity, PI3K activation, and MAP-kinase activation, show differential responsiveness to different receptor combinations, depending on which aspect of the activation process is considered. Thus, whereas heterodimers between EGFR and p185$^{erbB\text{-}2}$ have a moderate affinity interaction after stimulation by EGF (Karunagaran et al., 1996; Pinkas-Kramarski et al., 1996; Tzahar et al., 1996), EGFR/p185$^{erbB\text{-}2}$ is apparently not efficient in directly recruiting PI3K (Fig. 6). In contrast, EGFR/*erb*B-3 heterodimers are known to have an even lower affinity in cross-linking studies than EGFR/p185$^{erbB\text{-}2}$, yet our data now show that EGFR/*erb*B-3 does significantly recruit PI3K in response to stimulation by EGF (Fig. 6). Finally, as mentioned earlier, both EGF and HRG-β are known to strongly activate the MAP-kinase pathway by EGFR/EGFR and p185$^{erbB\text{-}2}$/*erb*B-3, respectively (Marte et al., 1995; Karunagaran et al., 1996; Pinkas-Kramarski et al., 1996).

By itself, *erb*B-3 is known to be almost completely kinase deficient (as would be expected from sequence analysis that shows alterations in the enzymatic site) and is thus not very potent in activating PI3K by itself in engineered cell lines that do not coexpress the other *erb*B kinases (Carraway and Cantley, 1994; Wallasch et al., 1995; Karunagaran et al., 1996; Pinkas-Kramarski et al., 1996; Tzahar et al., 1996). This indicates that the *erb*B-3 homodimer is not effective in activating signal transduction in the absence of the other *erb*B kinases but, rather, *erb*B-3 relies on heterodimer formation with the other *erb*Bs to effectively activate signal transduction pathways. Whereas the other *erb*Bs provide efficient kinase domains, *erb*B-3 contributes multiple additional docking sites for PI3K and other signaling molecules. Also, p185$^{erbB\text{-}2}$ is known to be an especially active kinase, even under ligand-independent conditions (Di Fiore et al., 1990; Lonardo et al., 1990; Peles et al., 1991, 1992; Janes et al., 1994). These combined considerations may account for the especially potent activation of signal transduction associated with p185$^{erbB\text{-}2}$/*erb*B-3 heterodimers in response to HRG-β, as well as to that seen in cancer cells with constitutively active p185$^{erbB\text{-}2}$ (Alimandi et al., 1995; Ram and Ethier, 1996; Siegel et al., 1999).

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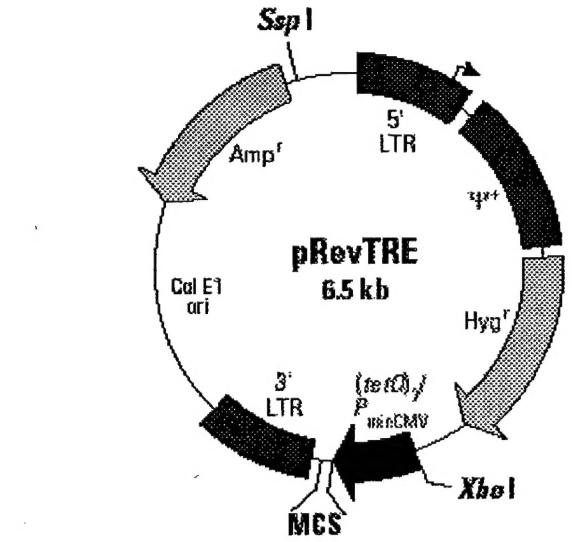

Restriction map and multiple cloning site (MCS) of pRevTRE Vector. All restriction sites shown are unique.

Note: The vector sequence file has been compiled from information in the sequence database, published literature, and other sources, together with partial sequences obtained by CLONTECH. This vector has not been completely sequenced.

The viral supernatants produced by this retroviral vector could, depending on your cloned insert, contain potentially hazardous recombinant virus. Due caution must be exercised in the production and handling of recombinant retrovirus. Appropriate NIH, regional, and institutional guidelines apply.

Description

pRevTRE is a retroviral Tet response vector that expresses a gene of interest from the Tet-response element (TRE). This vector is derived from pLNCX, a retroviral vector created using elements of Moloney murine leukemia virus (MoMuLV) and Moloney murine sarcoma virus (MoMuSV) as described (1). The TRE contains seven direct repeats of the *tetO* operator

sequence, upstream of a minimal CMV promoter, which can be bound by the tTA and rtTA transactivators. The 5' viral LTR controls expression of the transcript that contains Ψ^+ (the extended viral packaging signal) and the hygromycin resistance (Hyg^r) gene for antibiotic selection in mammalian cells. The TRE is derived from vectors described previously (2, 3). pRevTRE also includes the *E. coli* Amp^r gene for antibiotic selection in bacteria.

pRevTRE is provided with the control vector pRevTRE-Luc, which was constructed by cloning the firefly luciferase gene into the *Hin*d III/*Cla* I sites in the MCS of pRevTRE.

Vector	Size	Cat. #
pRev-TRE	20 µg	6137-1

Use

pRevTRE can be used to establish inducible Tet Systems via retrovirus-mediated gene transfer (4). Retroviral gene transfer allows the highly efficient transduction of virtually all dividing cell types. The RevTet™ Systems are also suitable for establishing transgenic animals. In combination with the pRevTet-On or pRevTet-Off regulatory vector, a gene of interest can be inducibly expressed at high levels in response to varying concentrations of tetracycline (Tc) or Tc derivatives such as doxycycline (Dox). tTA and rtTA bind to the Tet-response element (TRE) and activate transcription from the minimal promoter in the absence or presence of Dox, respectively. pRevTRE lacks the viral genes *gag*, *pol*, and *env*, which are supplied by the packaging cell line. It can be transfected into a high titer packaging cell line and thereby mediate production of infectious, replication-incompetent retroviral particles (1, 6–7). The transcript produced by the pRevTRE construct is recognized by the viral structural proteins expressed in a packaging cell line and packaged into infectious retroviral particles. Because the RNA transcript packaged in these particles does not contain the viral genes, it cannot replicate in the target cells that it infects.

The level of induction in cell populations infected with this vector depends on the efficiency of infection, the site of integration, and the titer of the virus. Viral supernatants with titers $>10^5$ cfu/ml should be produced to achieve high-level induction.

Location of Features

- 5' MoMuSV LTR: 1–589
- Ψ^+ (extended packaging signal): 659–1468
- Hygromycin resistance gene: 1510–2544
  Start codon: 1510–1512; stop codon 2542–2544
- TRE ($[tetO]_7/P_{minCMV}$): 2833–3276
- MCS: 3277–3354
- 3' MoMuLV LTR: 3390–3983
- Ampicillin resistance (β-lactamase) gene: 6139–5279
  Start codon: 6139–3137; stop codon 5277–5279

Propagation in *E. coli*

- Suitable host strains: DH5α, HB101, and other general purpose strains.
- Selectable marker: plasmid confers resistance to ampicillin (50 µg/ml) to *E. coli* hosts.
- *E. coli* replication origin: Col E1
- Copy number: low copy

References

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